

**Development of an shRNA screen to identify  
mediators of cellular senescence in human breast  
epithelial cells**

**By**

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***Author's declaration***

I, Katharina Wanek, confirm that the work presented in this thesis is my own.  
Where information has been derived from other sources, I confirm that this has  
been indicated in the thesis.

## **Abstract**

Cells from organisms with renewable tissues can permanently withdraw from the cell cycle in response to a variety of stimuli, including dysfunctional telomeres, DNA damage, physiological stress, and activation of certain oncogenes. This phenomenon, cellular senescence, is controlled by the p53 and pRb tumour suppressor proteins, and constitutes a potent anti-tumour mechanism. The underlying mechanism controlling cellular senescence and the signal transduction pathways involved are poorly defined and the critical targets of the p53 and pRb pathways in this process are not well characterised.

As most breast cancers originate in epithelial cells, Mike O'Hare, Parmjit Jat and colleagues developed a conditionally immortalized human mammary epithelial line, 226L 8/13, derived from human mammary luminal epithelial cells, as a model to study cellular senescence in epithelial cells. 226L 8/13 cells constitutively express hTERT, the catalytic component of human telomerase, and a temperature sensitive non-DNA-binding mutant of Simian Virus 40 large T (SV40 LT) antigen. These cells grow at the permissive temperature 34°C, but undergo a rapid growth arrest at the non-permissive temperature 38°C, upon inactivation of the thermolabile LT antigen which allows activation of the p53 and pRB tumour suppressor pathways. This arrest displays features of cellular senescence, and is dependent mainly on the p53 pathway, but also on the pRb pathway.

The Open Biosystems Human pGIPZ lentiviral shRNAmir library and pSM2c retroviral shRNAmir library were applied to the conditional system to identify mediators of cellular senescence, and several genes whose inhibition leads to cells overcoming the conditional growth arrest were identified, including IRF-1, FOXA1, Rab23 and CCNL1. By acting as mediators of cellular senescence these genes may play a role in the prevention of tumourigenesis. There is already significant evidence suggesting that IRF-1, FOXA1, Rab23 and CCNL1 play a role in cell proliferation, senescence and tumourigenesis, supporting the results presented in this thesis. Genes identified in this study may have prognostic and/or diagnostic value in the context of cancer biology.

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## ***Abbreviations***

AAK1	adaptor protein 2 associated kinase 1
AB	Applied Biosystems
Ago	Argonaute
ALT	alternate lengthening of telomeres
Amp <sup>R</sup>	ampicillin resistance gene
APC	anaphase-promoting complex
Apaf-1	apoptotic protease-activating factor-1
ARF	Alternative Reading Frame
ATM	ataxia-telangiectasia mutated
B2M	β 2-Microglobulin
BAMBI	BMP and activin membrane-bound inhibitor homolog
Bax	Bcl-2-associated X protein
BRCA1	breast cancer 1
BrdU	bromodeoxyuridine
CaCl <sub>2</sub>	calcium chloride
CALLA	Common acute lymphocytic leukemia antigen
CCNL1	Cyclin L1
CD95	cell-death signalling receptor
CDK	cyclin dependent kinase
CDKI	CDK-inhibitor
Ci	Cubitus interruptus
CSC	cancer stem cell
CSHL	Cold Spring Harbor Laboratories
C-terminal	carboxy terminal
Cul3	cullin 3
DAPI	4'-6-diamidino-2-phenylindole
Daxx	death-domain associated protein

DCIS	Ductal carcinoma in situ
DDI water	Distilled De-Ionized water
dd water	double-distilled water
DDR	DNA-damage response
DMSO	dimethyl sulphoxide
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
dNTPs	deoxyribonuclease triphosphate molecules
DR5	death receptor 5
DSB	double strand break
dsRNA	double-stranded RNA
E1A	early region 1A
E2F	E2 factor family
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EFs	Embryonic fibroblasts
ER	oestrogen receptor
ERBB3	epidermal growth factor receptor-related protein tyrosine kinase B3
EST	expressed sequence tag
EZH2	enhancer of zeste homologue 2
FLJ	full-length long Japan
FOXA1	forkhead box A1
GAP	GTPases activating protein
GDP	guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSE	genetic suppressor element
GTP	guanosine triphosphate

HDAC	histone deacetylase
HDF	human diploid fibroblasts
HEK 293 T/17	Human Embryonic Kidney 293 T/17 cell lines
HER-2	human epidermal growth factor receptor-2
Hh	hedgehog
HMEC	human mammary epithelial cell
HPV	Human papillomavirus
HRP	horseradish peroxidase
Hrs	hours
hTERT	catalytic subunit of human telomerase
IFN	interferon
IMS	industrial methylated spirit
IRF-1	interferon regulatory factor 1
KATNA1	katanin p60 subunit A 1
KLF	Krüppel-like transcription factor
LOX	lysyl oxidase
M1	mortality stage 1
M2	mortality stage 2
MAPK	mitogen-activated protein kinase
MaSCs	mammary stem cells
MCM7	minichromosome maintenance complex component 7
Mdm2	mouse double minute 2
MEFs	mouse embryonic fibroblasts
Mins	minutes
MOI	multiplicity of infection
MPF	M phase/maturation promoting factor
N2	liquid nitrogen
Nt	nucleotides

N-terminal	amino terminal
OD	optical density
OHT	4-hydroxy-tamoxifen
OIS	oncogene induced senescence
OB	Open Biosystems
PAI-1	Plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	dsRNA dependent protein kinase
pSM2c	pShagMagic2c
R	Restriction Point
Rab23	member RAS oncogenes family
REFs	rat embryonic fibroblasts
RNAi	ribonucleic acid interference
RNF135	ring finger protein 135
RIPA	radioimmunoprecipitation
RISC	RNA-induced silencing complex
rpm	revolutions per minute
RT PCR	reverse transcription-polymerase chain reaction
SASP	senescence-associated secretory phenotype
SA $\beta$ -gal	senescence-associated $\beta$ -galactosidase
SDFs	senescence-associated DNA-damage foci
SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1
SHAFs	senescence-associated heterochromatin foci
shRNA	short hairpin RNA
siRNA	small interfering RNA
SPOP	speckle-type POZ protein
SPOPL	speckle-type POZ protein like

SUMO	sumo small ubiquitin like modifier
SV40 LT	Simian Virus 40 Large T antigen
TAE	Tris-acetate-EDTA
TDLU	terminal ductal lobular unit
TE	Tris-HCl-EDTA
TGF- $\beta$	transforming growth factor- $\beta$
T <sub>m</sub>	melting temperature
TRF2	telomeric repeat binding factor 2
TRF2 <sup><math>\Delta</math>B<math>\Delta</math>M</sup>	dominant negative TRF2
Ts	temperature sensitive
TSSK2	testis-specific serine/threonine kinase 2
U19tsA58	temperature sensitive non-DNA-binding mutant SV40 LT
UTR	untranslated region
w/v	weight per volume
ZNF754	zinc finger protein 42

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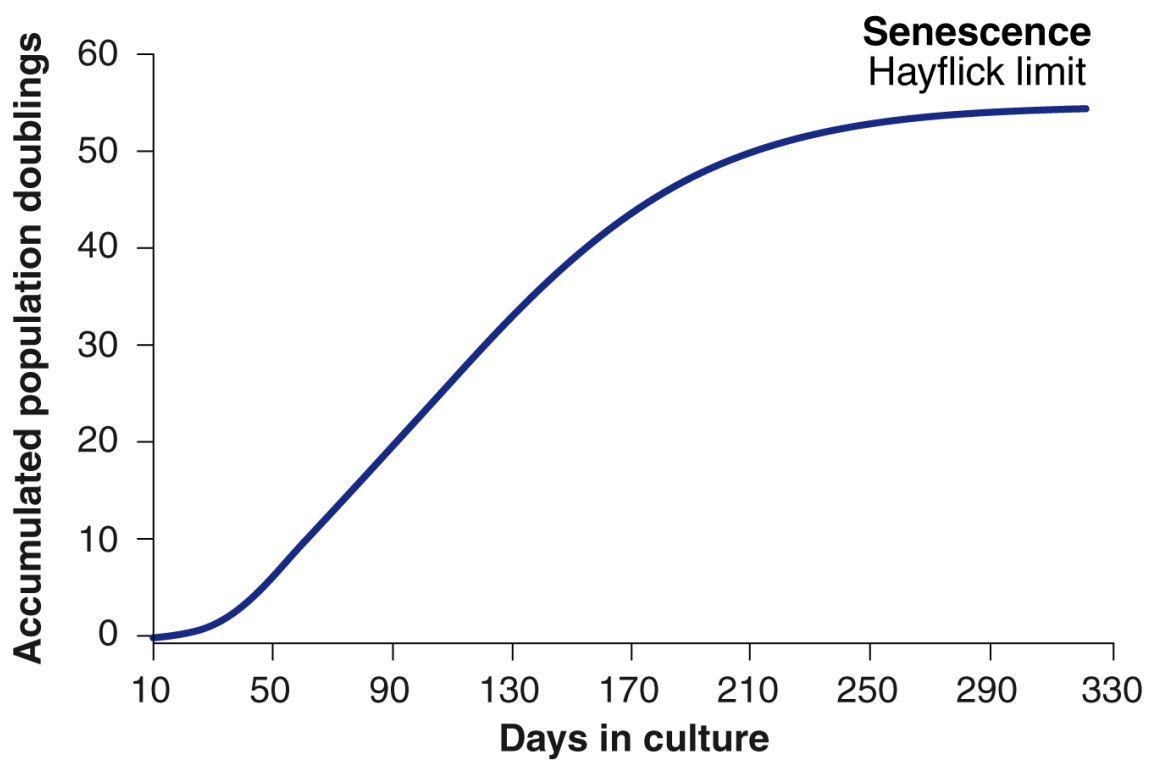
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## 1 Introduction

In 1961 Hayflick and Moorhead observed growth arrest of human diploid cells, after apparently exhausting their capacity to divide *in vitro* (Figure 1.1; (Hayflick and Moorhead, 1961)). They described this phenomenon as cellular senescence, and because they found an inverse correlation between proliferative potential of isolated cells and age of the donor, they assumed a central role of senescence in cellular and possibly organismal ageing. It has been suggested that pathological age of the donor might be more significant than chronological age (Cristofalo et al., 1998). Replicative senescence is the program of irreversible cell cycle arrest that normal cells undergo after a finite number of divisions (Hayflick and Moorhead, 1961), which is morphologically and biochemically closely related to premature senescence, an acutely inducible form of cellular senescence. Cellular senescence can be triggered in response to a variety of intrinsic and extrinsic stimuli including alteration in telomere length and structure (Hemann et al., 2001; Martens et al., 2000), deoxyribonucleic acid (DNA) damage (DiLeonardo et al., 1994), chromatin changes (Munro et al., 2004; Ogryzko et al., 1996), physiological stress such as sustained cytokine signalling (Moiseeva et al., 2006), and activation of certain oncogenes as first demonstrated by expression of Ras in human fibroblasts (Serrano et al., 1997). It can compromise tissue repair and regeneration and contribute to tissue and organismal ageing due to depletion of stem/progenitor cell compartments. It can also lead to removal of defective and potentially cancerous cells from the proliferating pool thereby preventing tumour development [reviewed by (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007)]. In the Hanahan and Weinberg model of tumorigenesis, overcoming the finite proliferative potential is one of the six hallmarks of cancer (Hanahan and Weinberg, 2011). The underlying signalling pathways that control cellular senescence and the signal transduction pathways involved are poorly understood.





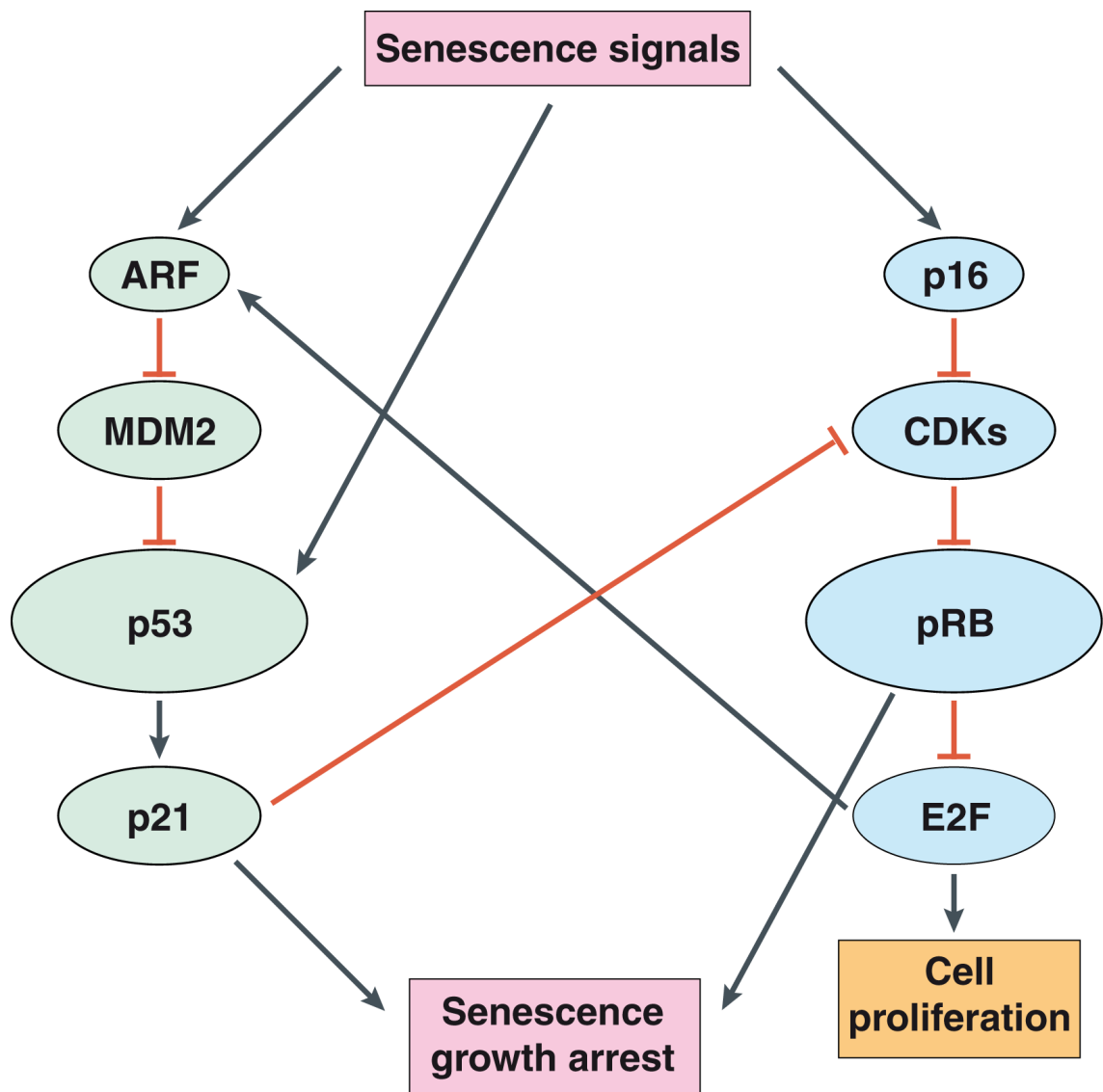
**Figure 1.1: Hayflick limit**

Hayflick and Moorhead (Hayflick and Moorhead, 1961) found that primary HDFs sub-cultivated in vitro exhibited a finite proliferative potential; after approximately 50 population doublings, at a point termed the 'Hayflick limit', the cultures ceased dividing and underwent an irreversible growth arrest, termed cellular senescence.

In this chapter I will discuss characteristics and causes of cellular senescence, the significance of senescence *in vivo* and its role in tumour suppression and ageing, senescence and its evolutionary context, and the acquisition of a limitless replicative potential. Also, I will discuss the cell cycle, specifically its role in the context of cellular senescence, and some DNA tumour viruses and their effect on the cell cycle. Furthermore I will discuss cell types of the human breast and their importance in cancer, and the establishment of conditionally immortalized human breast luminal epithelial cells, namely 226L 8/13 cells. Finally the concept of RNA interference (RNAi), short hairpin RNA (shRNA) libraries and their use for genetic screens will be introduced.

## **1.1 Cellular senescence**

Senescence can be divided into two broad categories, namely replicative senescence and premature senescence. Replicative senescence is the program of irreversible cell cycle arrest that normal cells undergo upon serial passage after a finite number of population doublings (Hayflick and Moorhead, 1961) believed to be caused by shortening of telomeres. Premature senescence is an acutely inducible form of cellular senescence, and is morphologically and biochemically strongly related to replicative senescence (Schmitt, 2007). Senescence stimuli activate the pRb and p53 pathway, which can in turn induce cellular senescence [Figure 1.2; reviewed by Campisi and d'Adda di Fagagna, 2007)]. In this chapter, features, causes and significance of cellular senescence will be discussed.



**Figure 1.2: Senescence stimuli activate the p53 and pRb pathways**

Senescence-inducing stimuli usually activate the p53 or/and the pRb pathway, which are the two main pathways underlying cellular senescence [Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Campisi and d'Adda di Fagagna, 2007), copyright (2007)]

### **1.1.1 Features of senescent cells**

#### ***1.1.1.1 Cellular senescence is an irreversible growth arrest***

The trademark of senescent cells is their inability to advance through the cell cycle. This growth arrest, in contrast to quiescence, is in essence irreversible; physiological mitogens do not stimulate growth in senescent cells, but senescence can be reversed by experimental manipulation. Expression of the tumour suppressor proteins p53 and pRb is strongly increased in senescent cells, and in senescent mouse embryonic fibroblasts (MEFs) inactivation of these two proteins leads to cells re-entering the cell cycle, which suggests that they are required for maintenance of senescence as well as induction of senescence in murine cells (Dirac and Bernards, 2003; Sage et al., 2003). In human cells it has been demonstrated that in cells that express p16<sup>INK4a</sup> at moderate to high levels, senescence cannot be reversed by the inhibition of p53, whereas in BJ cells, which express very low levels of p16<sup>INK4a</sup>, senescence can be reversed by p53 inactivation. In cells that express p16<sup>INK4a</sup> at moderate to high levels, suppression of p16<sup>INK4a</sup> confers sensitivity to senescence reversal by inactivation of p53 (Beausejour et al., 2003).

Senescent cells usually arrest growth with a DNA content characteristic of G<sub>1</sub> phase of the cell cycle, but it can also occur during G<sub>2</sub> phase (Di Micco et al., 2006; DiLeonardo et al., 1994; Olsen et al., 2002; Wada et al., 2004; Zhu et al., 1998), and in tumour cells in G-phase or S-phase when senescence is induced by certain cancer treatments (Shay and Roninson, 2004). Senescent cells stay metabolically active, demonstrated by their ability to synthesise mRNA and protein (Lumpkin et al., 1986).

#### ***1.1.1.2 Cellular senescence and apoptosis***

Senescent cells often become resistant to apoptosis (Seluanov et al., 2001; Wang, 1995; Yeo et al., 2000). Apoptosis is a controlled programme of cellular suicide; if cells die by apoptosis (as opposed to necrosis) the contents of dying cells are encapsulated and removed by scavenging cells. [reviewed by (Ellis et al., 1991)]. Like cellular senescence, apoptosis is a response to cellular stress and it is an important anti-tumour mechanism. Senescent cells can be resistant to certain apoptotic signals but sensitive to

others, and this resistance/sensitivity can be cell type specific. For instance, senescent human fibroblasts have been shown to enter apoptosis upon binding and activation of the Fas death receptor, but they have been demonstrated to be resistant to apoptosis induced by oxidative stress and growth factor deprivation (Chen et al., 2000; Tepper et al., 2000). Senescent human fibroblasts also resist ceramide-induced apoptosis, whereas endothelial cells are not resistant to it (Hampel et al., 2004). It is not yet clear what decides whether cells enter senescence or undergo apoptosis, but again, cell type seems to be a factor in this decision. For instance, damaged epithelial cells and fibroblasts usually enter senescence, whereas damaged lymphocytes tend to undergo apoptosis. Furthermore, it has been reported that manipulations of the expression levels of Bcl-2 or caspase inhibition may cause cells that normally would have undergone apoptosis to enter senescence (Nelyudova et al., 2007; Rebbaa et al., 2003). These studies indicate that there is crosstalk between the pathways of apoptosis and cellular senescence, possibly through their common factor p53 (Seluanov et al., 2001). It is not completely understood how cells become apoptosis resistant during *in vitro* senescence. This may partly be caused by the failure of senescent human fibroblasts to upregulate p53 after genotoxic stresses such as UV, actinomycin, cisplatin, or etoposide (Dimri et al., 1995; Seluanov et al., 2001). Another contributor for this apoptosis resistance of senescent cells is the high levels of the anti-apoptotic protein BCL-2 in senescent human fibroblasts (Wang, 1995).

#### ***1.1.1.3 Altered gene expression and senescence biomarkers***

Senescent cells have a changed gene expression profile compared to non-senescent cells. Certain markers, such as senescence-associated- $\beta$ -galactosidase [SA- $\beta$ -gal; (Dimri et al., 1995)], PAI-1, fibronectin (Kumazaki et al., 1991), p53 (Kulju and Lehman, 1995), and the cyclin dependent kinase inhibitors (CDKIs) p21 and p16<sup>INK4a</sup> (Alcorta et al., 1996; Hara et al., 1996; Tahara et al., 1995) have been shown to be up-regulated in senescent cells. On the other hand, positive regulators of the cell cycle have been shown to be down-regulated upon senescence, such as CDK2 (Afshari et al., 1993), cyclin A, cyclin B (Stein et al., 1991), cyclin E and cyclin D (Dulic et al., 1993). In senescent cells, E2F target genes can be down-regulated either because E2F is inactivated by pRb

and can not initiate transcription of such genes, or in other cases they are silenced by re-organization of chromatin into senescence-associated heterochromatin foci (SAHFs) caused by pRb (Narita et al., 2003).

Many of the proteins whose expression is altered upon senescence are secreted proteins, which is known as the senescence-associated secretory phenotype (SASP), or the senescence messaging phenotype secretome (Kuilman and Peeper, 2009). There is evidence that many of these factors not only correlate with senescence but also contribute to it. For example, the ECM regulator Plasminogen activator inhibitor-1 (PAI-1) was long believed to merely correlate with senescence, but was demonstrated to contribute to replicative senescence (Kortlever et al., 2006). The SASP includes proteins that are involved in remodeling the extracellular matrix (ECM) as well as numerous inflammatory proteins (Acosta et al., 2008; Cristofalo and Pignolo, 1996; Kuilman et al., 2008). There are three main groups of SASP factors, which are soluble signalling factors (interleukins, chemokines, growth factors), secreted proteases, and secreted insoluble protein/extracellular matrix components. In contrast to the tumour suppressive role of senescence, SASP has been demonstrated to promote carcinogenesis (Coppe et al., 2008; Krtolica et al., 2001; Kuilman and Peeper, 2009). These factors can promote tumour development *in vivo* and proliferation and invasiveness *in vitro* [reviewed by (Coppe et al., 2010)], as SASP factors can activate various receptors on the cell surfaces of surrounding cells, thereby activating certain signal transduction pathways which can contribute to cancer. These factors include interleukins, inflammatory cytokines, and growth factors. One example is the cytokine interleukin-6 (IL-6), which can affect various epithelial and endothelial cells which express the IL-6R (gp80) and gp130 signaling complex on their cell-surface.

The first senescence biomarker to be used was SA- $\beta$ -gal, which can be detected by histochemical staining in most senescent cells. This assay is based on the increased lysosomal biogenesis that occurs in many senescent cells (Lee et al., 2006), which therefore exhibit lysosomal  $\beta$ -galactosidase activity at pH6. Unfortunately, this marker can also be induced by stressful culture conditions and confluence in cell culture (Gary and Kindell, 2005; Iwasa et al., 2003).

Another marker frequently used is based on the fact that senescent cells contain many heterochromatin rich regions of DNA, known as SAHFs, which are believed to silence E2F target genes (Narita et al., 2003). These are dependent on hypophosphorylation of both pRb and p16<sup>INK4a</sup>. SAHFs can be detected by preferential binding of DNA dyes, for instance 4'-6-diamidino-2-phenylindole (DAPI).

p16<sup>INK4a</sup> is an important regulator of senescence, and it is also used as a marker for senescent cells (Krishnamurthy et al., 2004). Many senescent cells express p16<sup>INK4a</sup>, but there are exceptions such as senescent BJ cells (Beausejour et al., 2003; Itahana et al., 2003).

### **1.1.2 Senescence and terminal differentiation**

Complex organisms contain mitotic and post-mitotic cells. The loss of ability to divide in post-mitotic cells is caused by terminal differentiation (Potten and Lajtha, 1982; Till, 1982). Mitotic cells can proliferate, but they can also reversibly arrest, which is termed quiescence; quiescent cells re-enter the cell cycle in response to mitogenic signals. Cellular senescence only occurs in mitotic cells. There are similarities between senescence and growth arrest induced by terminal differentiation, as both cases cells stay viable but they undergo an irreversible growth arrest which makes cells resistant to mitogenic signalling (Wier and Scott, 1986). Furthermore, significant changes in morphology can be observed in both senescent and terminally-differentiated cells (Dimri et al., 1996) as well as changes in transcription, for instance induction of interleukins, cell cycle regulatory genes and DNA damage-inducible genes [reviewed by (Peacocke and Campisi, 1991)].

### **1.1.3 Cellular senescence and evolutionary antagonistic pleiotropy**

Cellular senescence can act as a anti-cancer mechanism, but on the other hand it can contribute to organismal ageing by depleting stem cell and progenitor cell pools, and it can even contribute towards tumourigenesis (Coppe et al., 2010). These contradictory actions of cellular senescence can be explained by a concept known as antagonistic pleiotropy; pleiotropic genes which are beneficial to an organism early in life will be

selected for, even if they have bad effects at later ages [reviewed by (Campisi, 2003; Campisi, 2005)]. For organisms with renewable tissues, cancer is a major threat to longevity; to counteract the risk of cancer, tumour-suppressor mechanisms evolved, such as cellular senescence and apoptosis (Hanahan and Weinberg, 2011). The detrimental actions of senescence are believed to be side effects of a mechanism which evolved for its beneficial effect on survival at a time when extrinsic hazards such as predation, infection and starvation caused life spans to be relatively short; therefore, tumour suppressor mechanisms functioned to extend lifespan at a relatively young organismal age and only needed to be beneficial in this relatively short life span, such as a few decades for humans. The fact that such mechanisms could be deleterious later in life, for example, by reducing the regenerative capacity and accumulation of dysfunctional senescent cells, would cause little selective pressure against these mechanisms.

#### **1.1.4 Causes of cellular senescence**

##### ***1.1.4.1 Telomere associated senescence***

Telomeres are DNA-protein complexes located at the ends of chromosomes; vertebrate telomeres are stretches of repetitive DNA (5'-TTAGGG-3') associated with proteins (Moyzis et al., 1988; Wellinger and Sen, 1997). Their main function is believed to be to protect the ends of chromosomes from being recognised as double-strand DNA breaks, which would result in degradation and end-to-end fusion with other chromosomes by DNA repair processes; for instance, recombination could occur through non-homologous DNA end-joining (Chan and Blackburn, 2002; Lundblad, 2000). The exact structure of telomeres is not known, but mammalian telomeres end in a large duplex loop, named t-loop (Griffith et al., 1999).

The unidirectional nature of DNA replication, 5' to 3', and the requirement for a primer to initiate DNA synthesis cause the 'end replication problem'. DNA can be replicated as a continuous piece of DNA on the 5' to 3' strand, known as the leading strand, but it has to occur in a discontinuous process on the lagging strand, producing DNA fragments known as Okazaki fragments. RNA primers are synthesised by the activity of primase,



an RNA polymerase that does not require a template for RNA polymerisation. DNA polymerase then replaces primase and polymerises DNA nucleotides to the RNA primer until it reaches the previously added RNA primer, which is then replaced with deoxyribonucleoside triphosphate molecules (dNTPs) by 5' to 3' exonuclease activity. Finally, these DNA fragments, Okazaki fragments, are then covalently linked by DNA ligase activity. But the last primer that is added to make the last Okazaki fragment at the 5' end of the lagging strand cannot be replaced by DNA, causing the 'end replication problem'. Therefore, about 50-200 base pairs of telomeric DNA is lost from the ends of the chromosomes during each S phase (Harley et al., 1990), causing telomeres to become shorter and eventually become critically short and dysfunctional. Only one or a few such short dysfunctional telomeres are sufficient to trigger senescence (Hemann et al., 2001; Martens et al., 2000).

The shortening of telomeres can be counteracted by elongation of telomeres by telomerase, which adds telomeric DNA repeats directly to the ends of chromosomes (Collins and Mitchell, 2002). This enzyme is composed of telomeric reverse transcriptase, hTERT (the catalytic subunit), an RNA component (TERC) which functions as the template for hTERT, and a number of associated factors which regulate its activity. In human somatic cells hTERT is not or hardly expressed, and if it is expressed then at levels that are so low they can merely slow down, but not prevent telomere shortening (Collins and Mitchell, 2002; Masutomi et al., 2003). However, it has been demonstrated that substantial levels of hTERT expression and telomerase activity occur in highly proliferating normal human cells and tissues, both *in vitro* and *in vivo* [reviewed by (Ge et al., 2006)]. Furthermore, hTERT expression and telomerase activity has also been demonstrated in normal cycling human diploid fibroblasts (HDFs), even though it was previously believed that in normal HDFs hTERT was transcriptionally repressed; it was shown that this telomerase activity was required for the maintenance of telomere structure, and that inhibition of telomerase activity leads to an increased rate of replicative senescence, and weakened DNA damage response in these HDFs [reviewed by (Ge et al., 2006)].

When telomeres become critically short they trigger senescence via initiation of the DNA-damage response (DDR) (d'Adda di Fagagna et al., 2003; Herbig et al., 2004;

Takai et al., 2003). There is evidence which demonstrates that telomere erosion can contribute to genome instability [reviewed by (Maser and DePinho, 2002)]. On the other hand, studies with mouse telomerase, p53 and p16<sup>INK4a</sup> knock-out models have shown that dysfunctional telomeres inhibit tumour initiation *in vivo* if an intact DNA damage-induced p53 signalling pathway is present, by activating either p53-dependent apoptosis or replicative senescence [reviewed by (Deng et al., 2008)]. These studies showed that in p16<sup>INK4a</sup> knock-out mice with short dysfunctional telomeres a reduced rate of tumour incidence was observed as compared to p16<sup>INK4a</sup> knock-out mice with functional telomeres upon treatment with ultraviolet B (Greenberg et al., 1999). But in p53 knock-out mice, telomere dysfunction and the consequential genomic instability promotes tumorigenesis, demonstrating that p53 cooperates with dysfunctional telomeres to reduce tumorigenesis.

Telomere shortening might function to generically avoid the growth of cancer, as telomere-induced senescence occurs when cells have undergone their maximum number of divisions, the Hayflick limit (Hayflick and Moorhead, 1961). Such proliferation-induced telomere shortening could make cancer self-limiting, and in many cancers telomerase is activated inappropriately (Counter et al., 1994) or other mechanisms that inhibit telomere shortening are activated, termed alternative lengthening of telomeres (ALT) mechanism (Muntoni and Reddel, 2005). Furthermore, it has been demonstrated that short telomeres can suppress tumour development in mice (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999), whereas expression of telomerase together with cooperating oncogenes can cause malignant transformation (Hahn et al., 1999).

#### **1.1.4.2 Oncogene-induced senescence**

The first indication of oncogene-induced senescence (OIS) came from *in vitro* studies, which demonstrated that oncogenic signalling can induce cellular senescence (Serrano et al., 1997). OIS is triggered by signalling by oncogenes, and it is accompanied by activation of tumour suppressor genes that are often inactivated in cancers, such as p16<sup>INK4a</sup> (Gruis et al., 1995b; Kamb et al., 1994). It therefore appears that OIS involves the activation of tumour suppressor genes. In many benign tumours proliferative arrest occurs, and it was speculated that oncogene-induced senescence might be responsible

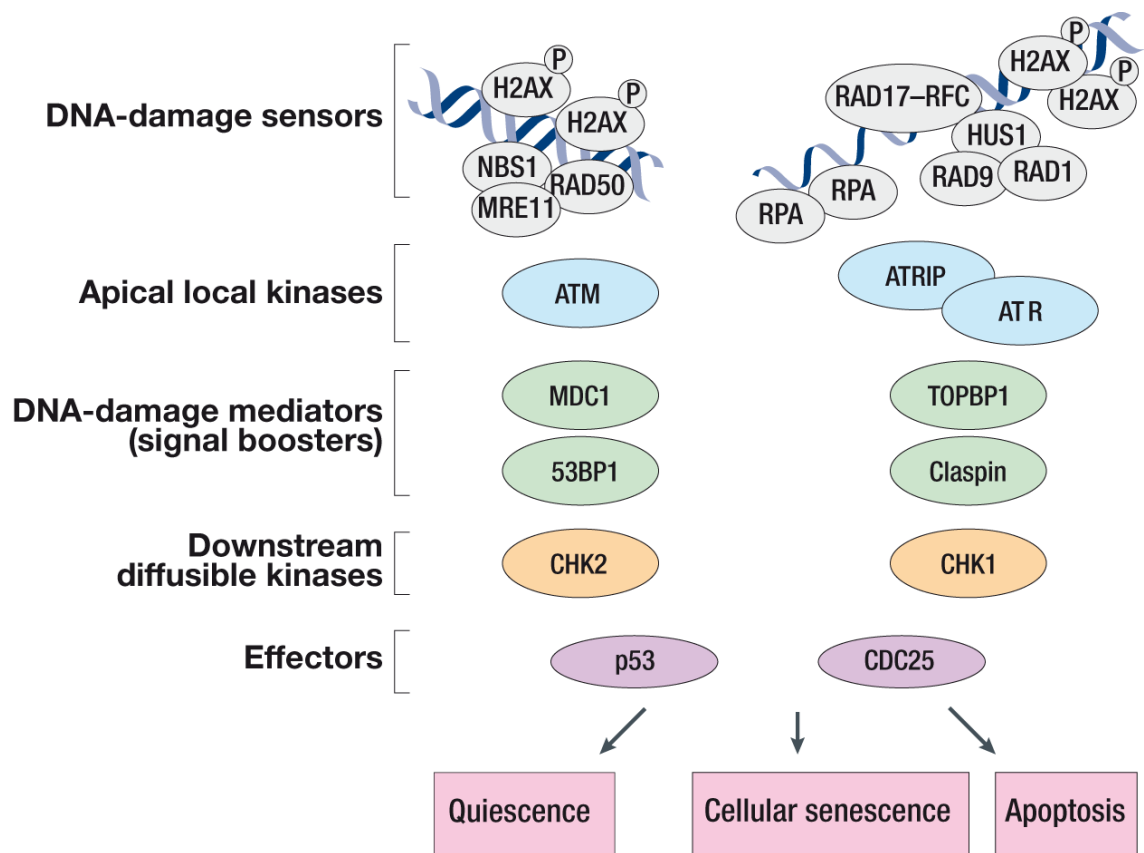
for this protective proliferation arrest; there is strong evidence supporting this theory (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Gray-Schopfer et al., 2006; Michaloglou et al., 2005).

Oncogenes induce cell proliferation, and the senescence response induced by OIS might be counteracting oncogenic transformation caused by the excessive proliferation induced by mitogenic stimulation. This excessive proliferation can lead to DNA damage, which in turn can induce a senescent arrest. This is supported by the finding that Ras-induced senescence is inhibited in mouse cells that are cultured in serum-free medium, which reduces mitotic signalling (Woo and Poon, 2004).

There are similarities between OIS and telomere induced senescence. Many oncogenes induce DNA-damage response, because the proliferation caused by excessive mitogenic stimulation by oncogenic signalling can lead to DNA damage which in turn induces DNA-damage response. It has been demonstrated that the DDR in OIS is required for induction and maintenance of senescence (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). These studies demonstrate that senescence, triggered by the expression of several oncogenes, is a consequence of the activation of a robust DDR and that experimental inactivation of DDR abrogates OIS and promotes cell transformation. Furthermore, both OIS induced by several oncogenes and telomere induced senescence lead to the activation of p16<sup>INK4a</sup> and cause the formation of SAHFS (Narita et al., 2003).

#### ***1.1.4.3 DNA-damage induced senescence***

DNA damage is sensed by the evolutionary conserved DDR pathway, starting with DNA-damage sensors, which activate a signalling cascade which leads to the induction of quiescence, senescence or apoptosis [Figure 1.3; reviewed by (d'Adda di Fagagna, 2008)].



**Figure 1.3: DNA-damage response and induction of cellular senescence**

DNA damage is sensed by the evolutionary conserved DDR pathway, starting with DNA-damage sensors, which activate a signalling cascade which leads to the induction of quiescence, senescence or apoptosis [Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (d'Adda di Fagagna, 2008), copyright (2008)].

As discussed above, the DNA-damage response is activated both in telomere-dependent and oncogene-induced cellular senescence, and it seems like cellular senescence pathways, whether replicative senescence or premature senescence that are induced by different stressors, share a common underlying mechanism, that is, DDR. *In vitro* cultured cells undergo irreversible growth arrest in response to various forms of DNA damage (Parrinello et al., 2003; te Poele et al., 2002). There is also evidence that accumulation of DNA damage due to ageing might contribute to cellular senescence (Vijg et al., 2005), leading to an accumulation of senescent cells in ageing tissues as well as depletion of stem cell and progenitor cell pools. Many chemotherapeutic drugs induce DNA damage, and studies show that these therapies are more effective in tumours which can senesce rather than those that do not senesce (Roninson, 2003; Schmitt et al., 2002).

Like in telomere-induced senescence, the p53 pathway plays an important role in DNA-damage induced senescence (d'Adda di Fagagna et al., 2003; DiLeonardo et al., 1994; Herbig et al., 2004). p16<sup>INK4a</sup> has also been shown to be induced in response to DNA damage and dysfunctional telomeres, and it is believed to function as an additional barrier to stop cells with damaged DNA or telomeres from re-entering the cell cycle (Beausejour et al., 2003; Jacobs and de Lange, 2004). P16<sup>INK4a</sup> knock-down alone was not sufficient to overcome a TRF2<sup>ΔBΔM</sup>-induced telomere-induced senescence arrest in IMR90 primary human fibroblasts; but p16<sup>INK4a</sup> did seem to contribute to the proliferation arrest as inhibition of p53 lead to a partial escape from TRF2<sup>ΔBΔM</sup>-induced growth arrest, whereas inhibition of both p16<sup>INK4a</sup> and p53 together led to a near complete restoration of the rate of DNA synthesis and an increased rescue from growth arrest ( Jacobs and de Lange, 2004).

#### ***1.1.4.4 Other causes and mediators of cellular senescence***

In addition to the fact that heterochromatin formation and SAHFs can induce cellular senescence, it seems like euchromatin formation can also induce senescence. Chemical histone deacetylation, which induces euchromatin formation, has been shown to promote senescence (Munro et al., 2004; Ogryzko et al., 1996). The fact that chemical histone deacetylation can induce ATM kinase activity (Bakkenist and Kastan, 2003), the

key kinase responsible for DDR double-strand break (DSB) signalling (Zgheib et al., 2005), suggests that euchromatin induced senescence might be involved in this process.

Sustained signalling of certain cytokines can also induce cellular senescence, and secreted inflammatory cytokines (Moiseeva et al., 2006), their cognate receptors and positive-feedback loops with corresponding transcription factors, have been implicated as key mediators of both oncogene-induced and replicative senescence (Bartek et al., 2008).

### ***1.1.5 Senescence and its relevance in vivo, tumour suppression and organismal ageing***

#### ***1.1.5.1 Identification of in vivo senescence by SA- $\beta$ -gal staining***

Dimri and colleagues (Dimri et al., 1995) first used SA- $\beta$ -gal staining to identify senescent fibroblasts and keratinocytes in ageing human skin *in vivo*. Since then it has been used to identify senescent cells in many other tissues. For instance, senescent cells were detected in human breast epithelial tumours following chemotherapy induced DNA damage (te Poele et al., 2002). Choi and colleagues detected SA- $\beta$ -gal-positive epithelial cells in prostates from men with benign hyperplasia (Choi et al., 2000).

#### ***1.1.5.2 P16<sup>INK4a</sup> and p14<sup>ARF</sup> and detection of in vivo senescence***

It has been shown that expression levels of the tumour suppressors p16<sup>INK4a</sup> and p14<sup>ARF</sup> correlate with SA- $\beta$ -gal positivity in rodents in organ pathology (Krishnamurthy et al., 2004) demonstrating the presence of senescent cells. P16<sup>INK4a</sup> expression levels have been demonstrated to increase during ageing in the human kidney (Melk et al., 2003; Melk et al., 2004). Benign melanocyte naevus cells express p16<sup>INK4a</sup> and other senescence markers such as SA- $\beta$ -galactosidase. This demonstrates that senescence occurs in such benign melanocytic naevi *in vivo* (Gray-Schopfer et al., 2006).

Furthermore, it has been shown that myocardial cells of pathological hearts from elderly patients have increased levels of p16<sup>INK4a</sup> as compared to non-pathological hearts from

patients of equal age or pathological hearts from younger subjects (Chimenti et al., 2003), demonstrating the increased presence of senescent cells in pathological hearts from elderly patients.

#### ***1.1.5.3 Telomeres and in vivo senescence***

Telomere shortening was first identified *in vitro* in human fibroblasts (Harley et al., 1990), and it has since been demonstrated *in vivo* in many tissues such as skin (Lindsey et al., 1991), the kidney (Melk et al., 2000), and the liver (Aikata et al., 2000). In human atherosclerosis, it was demonstrated that vascular smooth muscle cells undergo telomere-induced senescence (Matthews et al., 2006).

#### ***1.1.5.4 Oncogene induced senescence in vivo***

Since OIS was identified *in vitro* (Serrano et al., 1997), significant evidence has been produced which suggests a role in tumour suppression *in vivo* (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). RAS knock-in mice were shown to develop lung adenomas with a low proliferative rate, which expressed SA- $\beta$ -gal as well as other senescence markers (Collado et al., 2005). Michaloglou and colleagues (Michaloglou et al., 2005) Demonstrated that mutant oncogene BRAFE600 induces senescence-like arrest *in vitro* associated with SA- $\beta$ -gal activity in cultured human melanocytes. Validating these results *in vivo*, they confirmed the presence of the BRAFE600 mutation in eight specimens of a panel of 23 naevi, and high levels of SA- $\beta$ -gal in a panel of 23 naevi they analysed.

#### ***1.1.5.5 Senescence, organismal ageing and tumour suppression***

As discussed above, the use of senescent markers demonstrates the accumulation of senescent cells *in vivo*. They accumulate in multiple tissues and are often associated with pathologies, such as atherosclerosis. The accumulation of senescent cells may impair tissue repair and renewal due to a depletion of stem and progenitor cells. The secretory phenotype of senescent cells, SASP, includes proteins that are involved in

tissue environment (Acosta et al., 2008; Cristofalo and Pignolo, 1996; Kuilman et al., 2008), and can therefore alter functionality and structure of tissue which could contribute to the ageing phenotype. Furthermore, senescent cells, in contrast to their tumour-protective function, can promote carcinogenesis *in vivo* due to SASP and secretion of matrix degrading enzymes (Coppe et al., 2008; Krtolica et al., 2001; Kuilman and Peeper, 2009). On the other hand, senescence seems to be a protective mechanism against tumorigenesis. Evidence for this is the accumulation of cells expressing senescent markers *in vivo* in benign tumours, preventing the emergence of carcinoma (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Gray-Schopfer et al., 2006; Michaloglou et al., 2005). In contrast to normal somatic cells, cancer cells have the potential to proliferate indefinitely and this acquisition of an infinite proliferative potential has been proposed to be one of the six key events required for malignant transformation (Hanahan and Weinberg, 2011). These opposite effects of senescence suggest that ageing might be a consequence of antagonistic pleiotropy; senescence tumour suppression is beneficiary to an organism early in life and was therefore evolutionary selected for, even though it has negative effects at later ages. This is supported by studies such as that by Tyner and colleagues who developed a mouse with a mutant p53 allele that appears to enhance overall p53 activity, resulting in enhanced cancer resistance which is accompanied by premature ageing phenotypes and reduced longevity (Tyner et al., 2002).

## 1.2 The cell cycle

In complex, multicellular organisms, the ability of cells to divide is critical for both development and viability. The cell cycle and its checkpoints are involved in proliferation of cells, organismal development, maintenance of genomic stability, and cancer and tissue hyperplasia in response to injury. It is a complex process which ends in mitosis and the production of two daughter cells, and is controlled by numerous mechanisms to ensure the DNA of a cell is accurately replicated to maintain genomic stability in the daughter cells. Cyclin-dependent kinases (CDKs), which associate with and are regulated by cyclins, are very important in this process and guide cells through the cell cycle. Proteins such as p53, p21 and p16<sup>INK4a</sup> regulate cyclin proteins and



CDKs, and cyclin-CDK complexes regulate genes including pRb and E2F. The cell cycle can be divided into interphase, which consists of  $G_1$ , S, and  $G_2$  [reviewed by (Norbury and Nurse, 1992)], and M phase (mitotic phase), which consists of prophase, metaphase, anaphase, and telophase [reviewed by (Vermeulen et al., 2003)]; the cell cycle is illustrated in Figure 1.4. DNA replication occurs during S phase, which is preceded by  $G_1$ , which is when the cell prepares for DNA replication, and followed by  $G_2$ , which is when cells prepare for mitosis. During S phase cells have aneuploid DNA content between  $2N$  and  $4N$ . Cells in  $G_1$  before they are committed to DNA replication can become quiescent, which means they are not actively cycling, known as  $G_0$ .

### **1.2.1 The restriction point and cell cycle checkpoints**

The point at which a cell becomes committed to enter mitosis is termed Restriction Point [R, (Pardee, 1974)], which occurs two thirds of the way through the first gap phase ( $G_1$ ). Growth factors mainly affect cells in  $G_0$  and  $G_1$ . They induce the entry of cells from  $G_0$  to  $G_1$ , and before R in  $G_1$  cells return to  $G_0$  upon removal of growth factors. But once cells have reached R they no longer respond to withdrawal of growth factors and continue into S phase despite growth factor withdrawal. In addition to R there are also other checkpoints ensuring that the cell cycle occurs correctly (Hartwell and Weinert, 1989), namely DNA damage checkpoints and the spindle checkpoint. DNA damage checkpoints occur before the cell starts DNA replication ( $G_1 - S$  checkpoint) as well as after DNA has been replicated before the cell enters mitosis ( $G_2 - M$  checkpoint), and there are further DNA damage checkpoints during S and M phase.

### **1.2.2 Cell cycle regulation – cyclins, cyclin dependent kinases and their inhibitors**

CDKs are key regulators of the cell cycle, ensuring that the transition between the cell cycle phases occurs in orderly fashion. Several CDKs have been identified, these include CDKs active during  $G_1$  (CDK4, CDK6 and CDK2), during S phase (CDK2), and during  $G_2$  and M (CDK1) (Figure 1.4).

**Figure 1.4: The cell cycle** (*Figure has been removed due to Copyright restrictions.*)

The cell cycle can be divided into interphase, which consists of G1, S, and G2, and M phase. Different cyclin/CDK complexes are active during the different stages of the cell cycle [figure adapted from (Vermeulen et al., 2003)].

CDKs are a family of serine-threonine kinases which remain at relatively constant levels throughout the cell cycle, but they are activated at specific points during the cell cycle by cyclins.

Cyclins were the first regulators of the cell cycle to be discovered. They owe their name to the fact that they oscillate during early embryogenesis and drive the cell cycle in a cell autonomous manner; their levels oscillate during the cell cycle, and due to their falling and rising levels during the cell cycle they periodically activate CDKs (Evans et al., 1983; Pines, 1991). Since the discovery of cyclins many different cyclins have been identified, and they can be grouped according to when in the cell cycle they are required. For instance, the D-type cyclins are active in G<sub>1</sub>, and cyclin D-CDK complexes are essential for transit through G<sub>1</sub> (Sherr, 1994). Unlike other cyclins, cyclin D expression does not occur periodically but is induced by growth factor stimulation, and is not expressed in the absence of growth factors (Assoian and Zhu, 1997). Cyclins A and E are active during S phase and cyclins A and B during M phase.

The timing of expression of cyclins, their subcellular location and their biochemical activity are important for the cell cycle to occur in an orderly fashion [reviewed by (Murray, 2004)], and the regulation of CDK activity by cyclins ensures that CDKs act on their target proteins at the correct time during the cell cycle [reviewed by (Tessema et al., 2004)].

CDK activity is also regulated by phosphorylation of specific threonine and tyrosine residues. They can either have a positive effect on CDK function, as in the case of CDK1 phosphorylations caused by the cyclin H-CDK7 complex. These phosphorylations can lead to conformational changes which increase the binding of cyclins (Jeffrey et al., 1995; Paulovich and Hartwell, 1995). In other cases they can inhibit cyclin activity, such as phosphorylations of CDK1 caused by Wee1 and Myt1. In this case, dephosphorylation at the site phosphorylated by Wee1 and Myt1 is required for CDK1 activity and progression through the cell cycle [reviewed by (Lew and Kornbluth, 1996)].

Furthermore, CDKs are also regulated by CDK-inhibitors (CKIs). CKIs regulate cyclin-CKD complex activity by phosphorylating specific threonine, serine or tyrosine

residues in these complexes. There are two sub-groups of CDKIs, the Cip/Kip family and the INK4A family. The Cip/Kip family consists of the members p21, p27 and p57 [reviewed by (Sherr and Roberts, 1999)]. During the cell cycle, CDKs phosphorylate their target proteins to control their activity. During G<sub>1</sub> and G<sub>0</sub> cyclin levels are low and CDKI levels are high, leading to low CDK activity. Therefore, pRb, a CDK substrate, stays bound to the E2F transcription factor, preventing E2F from activating transcription of its target genes, which are required for entry into S phase. D type cyclins are active in the presence of mitogenic signalling; the level of D type cyclins can increase in response to extracellular signalling, which causes an increased level of cyclin D-CDK4/6 activity. This activity causes phosphorylation of the CDK substrate pRb, which in turn causes E2F to be released and activate its target genes [reviewed by (Bartek et al., 1996; Weinberg, 1995)]. Furthermore, cyclin D-CDK4/6 activity facilitates the expression of cyclin E. Cyclin E-CDK2 activity levels start to increase towards the end of G<sub>1</sub> phase, and they reach their highest level at the transition between G<sub>1</sub> and S phase (Dulic et al., 1992; Koff et al., 1992). The cyclin E-CDK2 activity can further increase phosphorylation of pRb, causing pRb to become fully inactivated. When S phase is reached cyclin A-CDK2 and cyclin E-CDK2 complexes are required (Coverley et al., 2002). Cyclin E stimulates replication complex assembly through interaction with Cdc6. Cyclin A-CDK2 promotes phosphorylation of components of the DNA replication machinery. Furthermore, they inhibit E2F, which is important to ensure that the cell cycle exits S phase to enter G<sub>2</sub>. Cyclin A-CDK2 stays active until the end of S phase. In the G<sub>2</sub> to M phase boundary cyclin B1 and cyclin B2 and their partner, CDK1, become active. They are components of the M phase/maturation promoting factor (MPF) that regulates processes that lead to assembly of the mitotic spindle and sister-chromatid pair alignment on the spindle. For exit of M phase degradation of cyclin B is required (Gallant and Nigg, 1992).

### **1.2.3 Rb family of proteins and pRb**

One of the main targets of cyclin-CDK activity is the pRb family of pocket proteins, which have in common a bipartite pocket region. These proteins are able to interact directly with proteins which possess an LXCXE domain, such as the histone

deacetylases HDAC1 and HDAC2, via specific residues in the pocket [reviewed by (Stiegler et al., 1998)]. Members of this family are pRb, p107, and p130, and their best known function is repression of transcription of E2F-regulated genes (Flemington et al., 1993; Frolov et al., 2001), but many more pRb binding proteins have been described [reviewed by (Morris and Dyson, 2001)]. Rb family members share common activities in the regulation of cell proliferation, differentiation and apoptosis (Claudio et al., 1996), but there is also functional specificity of individual members.

pRb is ubiquitously expressed, and its activity is regulated in a cell cycle-dependent manner which is in accordance with its function as a cell cycle regulatory protein [reviewed by (Cobrinik et al., 1992)].

The retinoblastoma gene (*RB1*) is one of the most intensively studied tumour suppressor genes. *RB1* mutations are involved in the development of the childhood cancer of the eye, retinoblastoma, and the product of the *RB1* gene, pRb, is believed to be absent or de-regulated in more than 90% of cancers; this includes mutations of *RB1* itself, as well as mutations in up-stream regulators such as homozygous deletion of p16<sup>INK4a</sup> or amplification of the CDK4 locus (Hanahan and Weinberg, 2011; Sherr and McCormick, 2002). Most tumour associated *RB1* mutations occur in the pocket protein domain of *RB1* (Hu et al., 1990; Huang et al., 1990). *RB1* was the first tumour suppressor gene cloned in humans, and it formed the basis of Knudson's two-hit hypothesis (Knudson, 1971). This hypothesis was supported by the results from analysis of patients with hereditary and non-hereditary forms of retinoblastoma. Knudson demonstrated that patients with hereditary retinoblastoma often developed bilateral tumours, whereas patients with the non-hereditary form usually only developed tumours in one eye. Knudson hypothesized that two mutational events are required for retinoblastoma to develop, but in patients that had the hereditary form one mutation was already present, therefore they only needed one mutational event to inactivate the remaining functional allele. Furthermore, familial tumours are likely to occur earlier since only one mutational event is required, whereas non-hereditary cancer requires two mutational events and therefore occurs later. Familial cancer often involves loss of heterozygosity, i.e. loss of normal function of one allele of a gene in which the other allele was already

inactivated, whereas non-hereditary cancer is usually caused by two independent mutations.

In  $G_0$  and early  $G_1$  the carboxy-terminal (C-terminal) domain of pRb is hypophosphorylated (Bonetto et al., 1999; Knudsen and Wang, 1996). This enables pRb to bind directly to E2F and inactivate it. It exerts its inactivating function on E2F in two ways; it binds to an 18 amino acid motif within the E2F transactivation domain, and thereby blocks the ability of E2F to form transcription activational complexes (Flemington et al., 1993; Helin et al., 1993a). In addition, pRb recruits repressive complexes such as histone deacetylase (HDACs) complexes and histone methyltransferases to the promoter region of E2F target genes to actively repress E2F transcription [reviewed by (Frolov and Dyson, 2004)]. pRb can spread the transcriptional silencing signal by binding to the heterochromatic protein HP1, which binds to modified histones and adjacent histone tails, thereby spreading silencing to nearby nucleosomes (Bannister et al., 2001; Lachner et al., 2001; Nielsen et al., 2001). This function of pRb causes the formation of a compact DNA structure which transcription factors cannot access. The fact that during  $G_1$  phase, pocket proteins can be detected together with E2Fs and histone deacetylase proteins in perinuclear foci, supports the role of pRb in transcriptional silencing (Kennedy et al., 2000).

Midway through  $G_1$  cyclin D1-CDK4/6 phosphorylates pRb, and at the restriction point R and late  $G_1$  phase, pRb is further phosphorylated by the activity of cyclin E-CDK2 [reviewed by (Adams, 2001)]. Late during  $G_1$  pRb hyperphosphorylation reaches its highest levels, which causes pRb to dissociate from E2F (Bonetto et al., 1999; Knudsen and Wang, 1996). Loss of Rb family repressor complexes at E2F-responsive promoters enables E2F to activate transcription of S phase genes which are required for DNA synthesis (Rayman et al., 2002; Takahashi et al., 2000; Taubert et al., 2004). pRb stays hyperphosphorylated until the transition to M phase, when it is dephosphorylated by PP1, a type 1 serine/threonine phosphatase (Nelson et al., 1997).

As well as regulation of the cell cycle, pRb is also involved in other processes such as senescence, differentiation and apoptosis. In senescence, E2F is bound to the pocket domain of pRb (Welch and Wang, 1995), and the pRb-E2F interaction leads to repression of E2F activity (Black and Azizkhan-Clifford, 1999; Helin et al., 1993a).

pRb not only exerts its growth suppression by inhibiting E2F activity, but it has also been shown that in some cases the pRb/E2F-1 complex can bind to promoters and actively inhibit transcription by blocking other transcription factors' activity on the promoters [reviewed by (Black and Azizkhan-Clifford, 1999).

In differentiation, pRb has been shown to interact directly with transcription factors to cause the differentiation of multiple cell lineages, such as adipogenesis, myogenesis and haematopoiesis (Condorelli and Giordano, 1997; Condorelli et al., 1995; Dunaief et al., 1994; Gu et al., 1993), and PRb is essential for normal mouse development, shown by the fact that homozygous mutants in the *Rb1* locus are embryonic lethals and have defects in neurogenesis and haematopoiesis (Lee et al., 1992).

Rb also seems to be involved in apoptosis. For example, it has been demonstrated that loss of E2F repression by Rb activity can induce caspase-8-mediated apoptosis (Lieman et al., 2005). Functional Rb activity has been demonstrated to inhibit Interferon- $\gamma$ -induced apoptosis (Berry et al., 1996)}.

#### **1.2.4 E2F**

The E2 factor family (E2F) of transcription factors act downstream of the pRb proteins and play an important role in cell cycle control. They were first discovered for their ability to bind to and activate the adenoviral E2 gene promoter (Kovesdi et al., 1986). E2Fs are known to have an important role in the positive regulation of genes required for entry into S phase and DNA synthesis, but functions in addition to G<sub>1</sub>/S control have been demonstrated. E2Fs have roles in both transcriptional activation and repression, and they have a role in many different processes such as proliferation, differentiation, apoptosis, tumour suppression and oncogenesis [reviewed by (Cam and Dynlacht, 2003; DeGregori, 2002). Genes regulated by E2Fs include genes involved in DNA replication, DNA repair and recombination, differentiation and development, mitosis, apoptosis and genes whose function is not yet known (Dimova et al., 2003; Ishida et al., 2001; Muller et al., 2001; Ren et al., 2002).

In mammals, eight *E2F* genes have been identified (E2F1-E2F8), and their protein products interact with other proteins to exert their many different functions. It has been

demonstrated that the mouse *E2F 3* gene codes for two E2F proteins, E2F3a and E2F3b (Leone et al., 2000). E2Fs act as heterodimers, and the proteins that E2Fs interact with are DP1, DP2 and DP3 (Dyson, 1998). For E2F activity, E2F and DP proteins heterodimerize, and it has been shown that they heterodimerize in all possible combinations *in vivo* (Bandara et al., 1993; Helin et al., 1993b; Wu et al., 1995). Furthermore E2Fs interact with the members of the pRb family (pRb, p107, and p130) (Dyson, 1998).

More is known about E2F1, E2F2, E2F3a, E2F4 and E2F5 than about the more recently discovered E2Fs, E2F6, E2F7, E2F8 and E2F 3b. The well studied members of the E2F family have varying functions and can be roughly divided into two functional groups [reviewed by (Trimarchi and Lees, 2002)]: E2F1, E2F2 and E2F3a oscillate during the cell cycle, they almost only interact with pRb and are positive regulators of transcription. The other group consists of E2F4 and E2F5, which are only weak transcriptional activators and their main function seems to be as transcriptional repressors of E2F regulated promoters by forming complexes with members of the pRb family. E2F4 can form complexes with all three pocket proteins, whereas E2F5 only interacts with p130 and p107 [reviewed by (Dyson, 1998)].

The less well studied members of the E2F family all seem to act as transcriptional repressors. Whereas E2F3a protein accumulates at the G<sub>1</sub>/S transition, E2F3b is constitutively expressed throughout the cell cycle, and it is the predominant E2F bound to target promoters in some quiescent cells (Chong et al., 2009; Leone et al., 2000). There is also evidence for E2F3b function in cycling cells, for instance E2F3b was shown to bind and repress the promoter of the p14<sup>ARF</sup> tumour suppressor under normal growth conditions (Aslanian et al., 2004).

E2F6 and E2F7 lack the transactivation domain and the pocket protein-binding domain that all the other E2Fs have in common, and they act as transcriptional repressors in a pocket protein-independent manner (Cartwright et al., 1998; de Bruin et al., 2003). E2F6 has been shown to act as a transcriptional repressor by binding to Polycomb group proteins (Trimarchi and Lees, 2002).



The most studied activity of E2F is its function in G<sub>1</sub> to S phase transition and initiation of DNA replication during S phase. E2F/Rb proteins transcriptionally regulate many genes required for S phase entry. Repressor E2F/pocket protein complexes are present in G<sub>0</sub> and in early G<sub>1</sub>, but they dissociate later in G<sub>1</sub> due to the phosphorylation of Rb proteins, to allow entry into S phase. When this occurs E2F activates transcription of its target genes [reviewed by (DeGregori, 2002; Trimarchi and Lees, 2002)]. E2F1, E2F2, and E2F3a function as transcriptional activators for E2F-target genes (Helin et al., 1992; Lees et al., 1993). Furthermore, if these transcription factors are over-expressed in quiescent cells it causes the cells to start cycling; E2F4 and E2F5 also have this effect but to a lesser extent (Johnson et al., 1993; Lukas et al., 1996). This process is dependent on the ability to bind DNA and activate transcription (Johnson et al., 1993). Established cell lines can be transformed by E2F1 overexpression, and in primary rat embryonic cells overexpression of E2F1 alone or together with activated Ras can cause oncogenic transformation (Johnson et al., 1994; Singh et al., 1994). In contrast, if E2F1 is overexpressed in primary human fibroblasts they enter a senescent-like state, which might be caused by p14<sup>ARF</sup> expression (Dimri et al., 2000).

Activating E2Fs seem to be required for proliferation. In primary cells anti-E2F3 antibodies can lead to cell cycle arrest (Leone et al., 1998). Furthermore, proliferation can be blocked completely if all 3 activating E2Fs, E2F1, E2F2 and E2F3, are mutated (Wu et al., 2001). Many possible E2F target genes are required in mitosis [reviewed by (DeGregori, 2002)]. They are involved in processes such as chromosome condensation, chromosome segregation, centrosome duplication, spindle checkpoints and cytokinesis. Hernando and colleagues demonstrated that in cells which have deregulated pRb/E2F function, the E2F target Mad2 is aberrantly expressed, which causes defects in mitosis and aneuploidy (Hernando et al., 2004).

The repressor E2Fs, E2F4 and E2F5, appear to be important in cell cycle exit and differentiation, and cells which do not have these E2Fs do not undergo G<sub>1</sub> arrest in response to some cell cycle arrest signals (Gaubatz et al., 2000).

There is evidence that pRb/E2Fs are directly involved in DNA replication. It has been demonstrated that Rb can bind to and inhibit certain proteins involved in DNA replication, such as DNA polymerase alpha (Takemura et al., 1997) and MCM7 to

inhibit replication (Sterner et al., 1998). Furthermore, Rb and E2F have been shown to be present at sites of DNA replication in S phase in mammalian cells (Kennedy et al., 2000; Lai et al., 2001). Also, pRb can be detected at replication initiation sites caused by DNA damage (Avni et al., 2003).

E2F might also be involved in DNA repair and in checkpoint control. Many genes that are possible targets of E2F have functions in DNA damage repair and in DNA damage checkpoints (Ren et al., 2002). There are studies which support a role of E2F in DNA damage response, for example it has been demonstrated that E2F1 protein levels rise and E2F activity increases in cells in which DNA damage is induced (Huang et al., 1997; Lin et al., 2001; Stevens et al., 2003). Severe DNA damage can lead to apoptosis, and it has been shown that E2F1 can induce apoptosis in reaction to DNA damage (Huang et al., 1997; Stevens et al., 2003), and in some cases E2F1 activity is required to induce apoptosis by DNA damage (Lin et al., 2001). It appears that E2F induced cell death can be p53-dependent (Qin et al., 1994; Wu and Levine, 1994) or a p53-independent (Holmberg et al., 1998).

### **1.2.5 The p53 pathway**

P53 is a member of a unique protein family which is comprised of 3 proteins, p53 (Deleo et al., 1979), p63 (Schmale and Bamberger, 1997) and p73 (Kaghad et al., 1997). The family members are structurally related to each other, but in higher organisms it appears that p53 has evolved to act as a tumour suppressor, whereas p63 and p73 function in organismal development [reviewed by (Irwin and Kaelin, 2001a; Irwin and Kaelin, 2001b)]. P53-null mice are prone to develop spontaneous and induced tumours (Donehower et al., 1992), whereas p63- and p73-null mice do not show increased tumorigenesis but instead developmental defects [(Mills et al., 1999); reviewed by (Irwin and Kaelin, 2001b)]. Nevertheless, p63 and p73 share some of the tumour suppressive functions with p53: All three members can homo-oligomerise, bind to DNA and act as transcriptional activators at p53 responsive promoters [(Osada et al., 1998; Yang et al., 1998)]; reviewed by (Irwin and Kaelin, 2001a)]. It has been shown that overexpression of p63 or p73 can induce transcription of p53 target genes and apoptosis (Irwin and Kaelin, 2001a; Jost et al., 1997; Yang et al., 1998), and that like abrogation

of p53, abrogation of p63 or p73 activity can inhibit apoptosis in response to DNA damage (Flores et al., 2002; Irwin and Kaelin, 2001a).

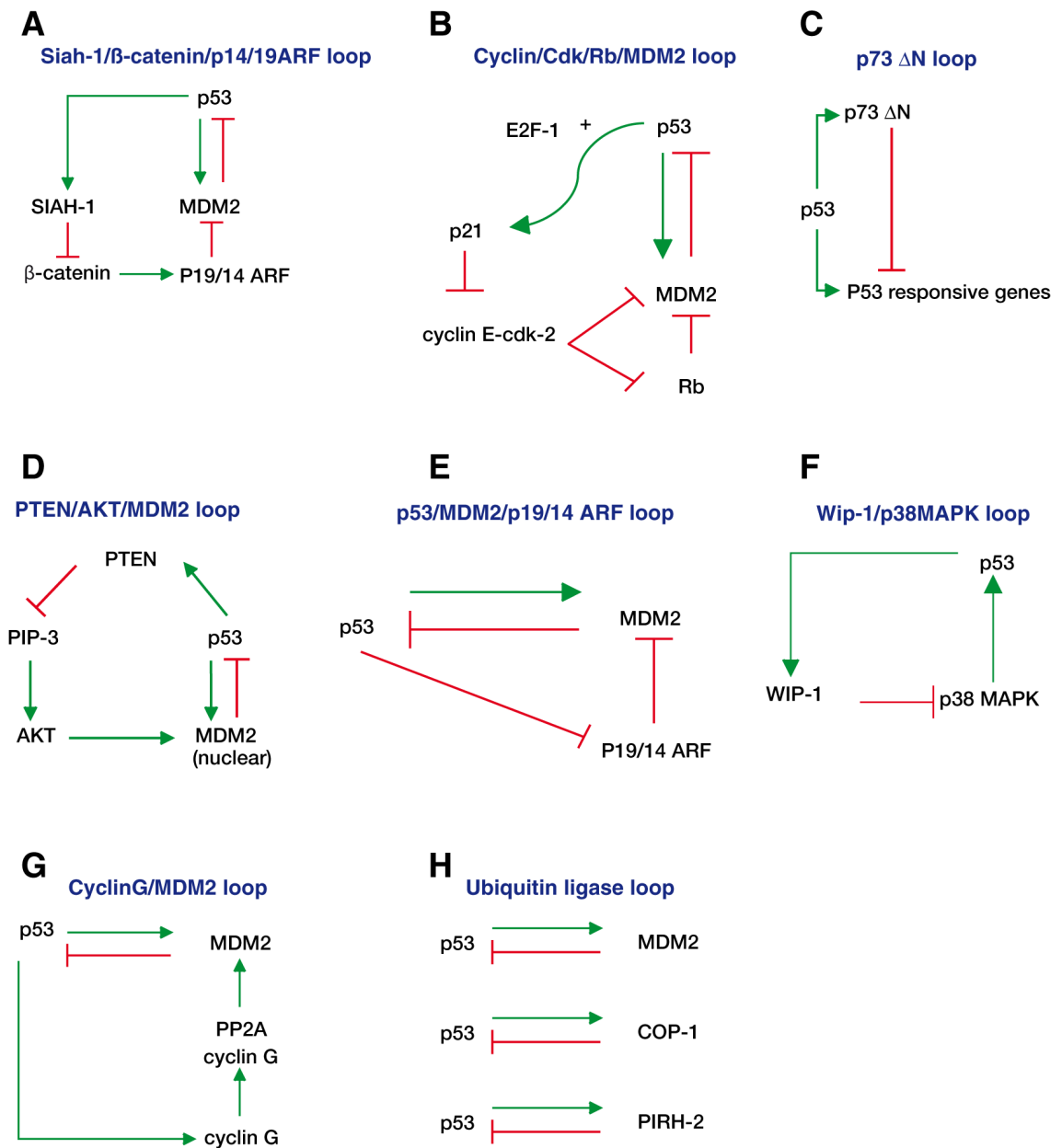
### **1.2.5.1 P53**

The nuclear phosphoprotein p53 was first identified as a protein that formed a complex with SV40 LT (Lane and Crawford, 1979; Linzer and Levine, 1979). It was found to be up-regulated in cancer and believed to act as an oncogene, but studies in knock-out mice demonstrated that p53 has a potent function as a tumour suppressor (Donehower et al., 1992), and following genotoxic stress it is vital to prevent inappropriate proliferation and to maintain genome integrity [reviewed by (Vogelstein et al., 2000; Vousden et al., 2002; Vousden, 2002)]. It has been demonstrated that p53 induces apoptosis or cell cycle arrest and DNA repair in response to DNA damage [reviewed by (Jin and Levine, 2001; Vogelstein et al., 2000)]. The *p53* gene is one of the most common sites of genetic mutations in human cancers (Hollstein et al., 1991; Levine et al., 1991), about half of human cancers have a mutated p53, and nearly all human cancers have de-regulated p53 function (Hollstein et al., 1994). p53 is important in controlling and integrating signals responsible for cell cycle control, and is therefore termed ‘guardian of the genome’ (Lane, 1992).

P53 is ubiquitously expressed at a low level, but in response to stress, such as DNA damage, heat shock, hypoxia or deregulated growth signalling it is activated and stabilized [reviewed by (Enoch and Norbury, 1995; Levine, 1997)]. When p53 is activated, p53 protein levels rise and extensive posttranslational modifications occur, which lead to qualitative changes in the protein, and this results in activation of p53 target genes (Fritsche et al., 1993). These posttranslational changes include phosphorylation, methylation, sumoylation and acetylation, which lead to changes in p53 stability and conformation, and in turn to changes in interactions with binding partners as well as sub-cellular location [reviewed by (Woods and Vousden, 2001)]. For instance, in response to DSB DNA damage ATM (ataxia-telangiectasia mutated) protein kinase becomes active which causes activation of chk2 kinase (Matsuoka et al., 1998). P53 is then phosphorylated by ATM and Chk2 at specific sites which in turn leads to cell cycle arrest or apoptosis (Banin et al., 1998; Canman et al., 1998). Phosphorylation of threonine residue 18 or serine residue 20 in the amino-terminus of p53 stabilises p53;

phosphorylation at these sites inhibits the negative action of Mdm2 on p53 (Kussie et al., 1996), which is a ubiquitin E3 ligase that blocks transactivation or repression of p53 target promoters by p53 by directly binding to its transactivation domain (Chen et al., 1995), and promotes p53 ubiquitination (Haupt et al., 1997; Kubbutat et al., 1997). Mdm2 relocates p53 into the cytoplasm, where ubiquitinated p53 is then targeted for proteolysis (Momand et al., 2000). Mdm2 is activated by p53, and it forms a negative feedback loop acting as a major negative regulator of p53 [reviewed by (Harris and Levine, 2005)]. If p53 dissociates from Mdm2 it returns to the nucleus and its half-life increases, which leads to a 3-10 fold increase in p53 levels in the nucleus [reviewed by (Harris and Levine, 2005)].

P53 proteins form tetramers, and they bind to DNA in a sequence specific manner to the p53 responsive element, the consensus binding sites consisting of two copies of a 10 base pair motif separated by 0-13 base pairs (Eldeiry et al., 1992). Many gene promoters have been shown to have a p53 responsive element, and genes whose transcription is activated by p53 include *MDM2*, *BAX* and *p21* (Hoh et al., 2002). Several positive and negative feedback loops have been identified in the p53 pathway (Figure 1.5). These loops contain proteins whose expression or activity is affected by p53 activation, and which in turn have an effect on p53 levels and activity [reviewed by (Harris and Levine, 2005)]. Eight of these loops are negative feedback loops (p14/19 ARF, MDM-2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1 and Siah-1), and two of them are positive feedback loops (PTEN-AKT and Rb). Six of the loops act via Mdm2 to affect p53 activity; these are the MDM-2, cyclin G, Siah-1, p14/19<sup>ARF</sup>, AKT and Rb loops.



**Figure 1.5: Positive and negative feedback loops of p53 signalling**

Several positive and negative feedback loops have been identified in the p53 pathway. These loops contain proteins whose expression or activity is affected by p53 activation, and which in turn have an effect on p53 levels and activity. Eight of these loops are negative feedback loops (A, C, E, F, G, H), and two of them are positive feedback loops (B and D) [Adapted by permission from Macmillan Publishers Ltd: [Oncogene] (Harris and Levine, 2005), copyright (2005)].

In addition to acting as a transcriptional activator p53 has also been shown to act as a transcriptional repressor. Genes such as *c-FOS* (Ginsberg et al., 1991), and *BCL2* (Miyashita et al., 1994) have been shown to be negatively controlled by p53. P53 might indirectly interact with other promoter-bound transcription factors (Horikoshi et al., 1995; Ragimov et al., 1993) as well as with histone deacetylases (HDACs), recruited by the co-repressor Sin3A (Murphy et al., 1999), to achieve this transcriptional repression.

Activation of p53 can lead to cell cycle arrest in G<sub>1</sub>, G<sub>2</sub>, and in S phase (Agarwal et al., 1995; Agarwal et al., 1998). When cell cycle arrest is induced at G<sub>1</sub> or G<sub>2</sub> due to DNA damage it ensures that DNA can be repaired before the cell enters S phase or mitosis. P53 also facilitates DNA repair, such as base excision repair and nucleotide excision repair, and once DNA has been repaired the cell can continue to cycle (Zhou et al., 2001). In response to cellular stress p53 upregulates the CDKI p21, which is the most important downstream target of p53 in DNA damage induced G<sub>1</sub> arrest (Eldeiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). P21 inhibits phosphorylation of pRb by cyclin E-CDK2, which in turn blocks E2F release from pRb and thereby the induction of genes which are required to enter S phase [reviewed by (Sherr and Roberts, 1999)]. Agarwal and colleagues have also demonstrated a p53-induced S phase checkpoint which is independent of p21 (Agarwal et al., 1998).

P53 has been shown to be involved in the induction of cellular senescence. If p53 function is lost in human cells, replicative senescence is delayed or abrogated [reviewed by (Itahana et al., 2001)]. Furthermore, p53 is essential for the induction of senescence by DDR [reviewed by (Wahl and Carr, 2001)]. It has also been shown that introduction of wildtype p53 into p53-null tumour cells can induce senescence in these cells (Sugrue et al., 1997). In telomere-induced senescence p53 has been shown to co-localize with components of the DNA damage signalling pathway at telomeres (d'Adda di Fagagna et al., 2003; Gire et al., 2004; Sugrue et al., 1997). In addition to induction of senescence, p53 also seems to have a role in maintenance of senescence (Atadja et al., 1995; Kulju and Lehman, 1995), and if senescent HDFs are injected with p53 antibodies to abrogate p53 activity, senescence can be temporarily reversed and cells can re-enter the cell cycle; cells enter S phase and mitosis as demonstrated by an increase in cell number (Gire and Wynford-Thomas, 1998). In BJ cells, which express very low levels of

p16<sup>INK4a</sup>, senescence can be reversed by p53 inactivation. In cells that express p16<sup>INK4a</sup> at moderate to high levels, suppression of p16<sup>INK4a</sup> confers sensitivity to senescence reversal by inactivation of p53 (Beausejour et al., 2003)

P53 can induce both quiescence and senescence, and it is unclear how the decision between the two responses is made. Recent findings indicate that levels of p53 might contribute to this choice [reviewed by (Meek, 2010)]. If cell cycle arrest is induced, hyper-active mTOR leads to cellular senescence. In some cell types, high levels of p53 can inhibit mTOR signalling; by this mechanism, high levels of p53 could lead to the induction of quiescence instead of senescence, whereas low levels of p53 could induce senescence.

In addition to induction of reversible cell cycle arrest, accompanied by DNA repair, and induction of senescence, p53 can also induce apoptosis in response to cellular stress (Chen et al., 1996b). Apoptosis is induced when damage is too severe to be repaired, and it is induced by p53 by several cellular stressors, such as DNA damage, hypoxia and growth factor deprivation (Soengas et al., 1999). Transcription of many apoptotic proteins is induced by p53, including Bax [Bcl-2-associated X protein; (Miyashita and Reed, 1995)], DRAL (Scholl et al., 2000), Fas/CD95 [cell-death signalling receptor; (O'Connor et al., 2000)], Apaf-1 [apoptotic protease-activating factor-1; (Moroni et al., 2001)] and DR5/KILLER [death receptor 5; (Takimoto and El Deiry, 2000)]. P53 induces proteins that are involved in both, the intrinsic pathway (the mitochondrial pathway), e.g. Bax, or the extrinsic pathway (the death receptor pathway), e.g. DR5/KILLER. One hypothesis is that p53 has the ability to activate apoptosis via multiple pathways because of the selective pressure to lose pro-apoptotic genes during tumorigenesis.

#### **1.2.6 P21<sup>Waf1/Cip1/Sdi1</sup>**

P53 transactivates various growth inhibitory or apoptotic genes in response to cellular stress. One of these genes is p21, which mediates p53 induced G<sub>1</sub> growth arrest (Eldeiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). P21 belongs to the Cip/Kip family of CDKIs which consist of the members p21, p27 and p57 [reviewed by (Sherr and

Roberts, 1999)]. P21 inhibits the kinase activity of cyclin-CDK complexes by binding to their amino-terminal (N-terminal) homologous sequences, and it regulates the activity of cyclin-CDK complexes by interfering with phosphorylation of CDK1 and CDK2 in the activation segment (Abbas et al., 2007; Mandal et al., 1998; Smits et al., 2000). P21 binding to cyclin is via a conserved Cy1 motif in the N-terminal domain, and it binds to the CDK subunit of the complexes through a CDK-binding site, also in the N-terminal domain (Chen et al., 1996a). By binding to the Cy motif p21 inhibits binding of cyclin-CDK complexes to substrates, such as Rb family proteins (Shiyanov et al., 1996; Zhu et al., 1995), thereby inhibiting Rb phosphorylation and subsequent release of E2F. P21 seems to inhibit cell cycle progression primarily by inhibiting activity of CDK2, thereby inhibiting CDK2 mediated phosphorylation and inactivation of Rb, as well as CDK2 mediated firing of replication origins and activation of proteins directly involved in DNA synthesis (Zhu et al., 2005). P21 affects DNA replication and DNA repair by competing for binding to PCNA (Floresrozas et al., 1994; Waga et al., 1994), a component of the DNA replication machinery [reviewed by (Moldovan et al., 2007)]. For example, p21 interferes with PCNA-DNMT1 binding (Chuang et al., 1997), which is required for DNA synthesis and repair (Mortusewicz et al., 2005; Walsh and Xu, 2006). Following DNA damage, once DNA has been repaired, p53 levels fall, which leads to p21 levels falling which in turn causes CDKs to become active and the cell can re-enter the cell cycle.

The view that CDK2 and inhibition of CDK2 by p21 plays a central role in cell cycle control has been challenged; there is evidence suggesting that proliferation of some human cancer cells is not dependent on CDK2 (Tetsu and McCormick, 2003). Furthermore, mice lacking this kinase develop normally (Berthet et al., 2003; Ortega et al., 2003) and cells lacking CDK2 proliferate well in culture and re-enter cell cycle without significant delay following serum starvation (Ortega et al., 2003). Furthermore, it has been shown that p21 and p27 can still block cell cycle progression in CDK2 deficient cells (Martin et al., 2005), demonstrating that these CDKIs inhibit cell cycle progression not only through CDK2 inhibition. It appears that in some tissues p21 might exert its anti-proliferative effects in tumorigenesis through CDK1 [reviewed by (Malumbres and Barbacid, 2009)]. Furthermore it has been demonstrated that p21 can inhibit both Cdk4 and Cdk2 activities to induce a G1 arrest (He et al., 2005). As p21 can



interact with a variety of cyclin/CDK complexes it can induce cell cycle arrest at any time , whereas the INK4 family specifically inhibits CDK4 and CDK6 and can only induce arrest in G1/G0.

There is substantial evidence that p21 can promote anti-proliferative effects independently of p53, for example p21 transcription can be activated by some nuclear receptors, such as androgen receptors and retinoid receptors, which bind to their cognate response elements in the p21 promoter to activate transcription (Gartel and Tyner, 1999). Several members of the Krüppel-like transcription factor (KLF) family can transactivate p21 in a p53 independent manner, for example KLF6 (Li et al., 2000). KLF6 is a tumour suppressor which is inactive or downregulated in many tumours, for example prostate cancer (Chen et al., 2003) and non-small cell lung cancers (Ito et al., 2004).

One of the ways in which p21 was originally identified was as an up-regulated gene in senescence (Noda et al., 1994) and it is thought to be one of the key regulators of senescent cells as demonstrated by the finding that inactivation of p21 was sufficient to bypass senescence in normal diploid human fibroblasts (Brown et al., 1997). There is significant evidence demonstrating the importance of p21 in cellular senescence. For instance, the p53-p21 pathway has been shown to be required for cellular senescence induced by the inhibition of protein kinase CKII in human colon cancer cells (Kang et al., 2009). P21 is also required for senescence induced by the loss of Apc in the renal epithelium (Cole et al., 2010). P21 accumulation upon senescence is transient, and expression of p21 falls again after the induction of senescence (Stein GH, 1998).

P21 mediates the growth repressive activity of p53, but it does not mediate p53-induced apoptosis (Attardi et al., 1996). Rather, through inducing reversible cell cycle arrest in response to certain stimuli, such as genotoxic stress, it protects cells from apoptosis as cells need to be actively cycling to trigger apoptosis in response to these stimuli. P21 contains a caspase cleavage site in its carboxy-terminal domain which is cleaved during induction of apoptosis, thereby stopping p21 from inducing growth arrest (Zhang et al., 1999b). The ability of p21 to inhibit apoptosis might explain its contradictory oncogenic activities (Roninson, 2002).

In contrast to its antiproliferative activities, p21 exerts vital pro-proliferative and pro-survival activities when it is localized in the cytosol. For example, when expressed at low levels p21 can act as an assembly factor for CDK4 and CDK6 with D type cyclins, and it also facilitates their transport to the nucleus and prevents them from being exported from the nucleus [reviewed by (Child and Mann, 2006)]. By this action it causes increased cyclin D/CDK activity, which leads to phosphorylation and inactivation of pRb, thereby allowing E2F induced transcription and progression through the cell cycle. Cytoplasmic p21 can also activate cyclin B/CDK activity, and it can block Fas-mediated apoptosis by binding to and inhibiting procaspase 3 [reviewed by (Child and Mann, 2006)]. P21 subcellular location can be controlled by phosphorylation of certain residues in the protein [reviewed by (Child and Mann, 2006)].

#### **1.2.7 P27**

P27 was first identified in complexes with cyclin E-CDK2 in transforming growth factor- $\beta$  (TGF- $\beta$ ) arrested cells (Koff et al., 1992). Like p21, p27 belongs to the Cip/Kip family of CDKIs which inhibit cell cycle progression, and like the other members of this family it inhibits cyclin-CDK activity by binding these complexes with its N-terminal domain, and by physically obstructing the catalytic cleft of the CDK, thereby blocking ATP binding (Russo et al., 1996). It has been shown that p27 can inhibit recombinant cyclin D-CDK complexes *in vitro*, but its inhibitory action on cyclin E-CDK2 activity is stronger (Polyak et al., 1994b; Toyoshima and Hunter, 1994). Cyclin E-CDK2 complexes can cause their own activation by phosphorylating p27 on a specific threonine residue to induce its degradation (Sheaff et al., 1997; Vlach et al., 1997). In addition to its negative regulation of cyclin-CDK activity, p27 also seems to facilitate assembly of cyclin D1/D2-CDK4 complexes, as their assembly has been shown to be impaired in MEFs lacking p27 (Cheng et al., 1999). P27 activity is regulated through transcriptional, translational and post-translational mechanisms (Bagui et al., 2009; Carrano et al., 1999; Hengst and Reed, 1996). P27 activity is induced by various signalling pathways, and factors that increase p27 include for example TGF- $\beta$  (Polyak et al., 1994a) and cAMP (cyclic adenosine mono- phosphate)

signalling (Shin et al., 2009). Some signalling pathways reduce its activity by phosphorylation, thereby allowing cell cycle progression (Jin et al., 2009; Morishita et al., 2008). P27 has been shown to play a significant role in regulation of the restriction point. In fibroblasts, inhibition of p27 prevents cell cycle arrest caused by mitogen depletion (Coats et al., 1996).

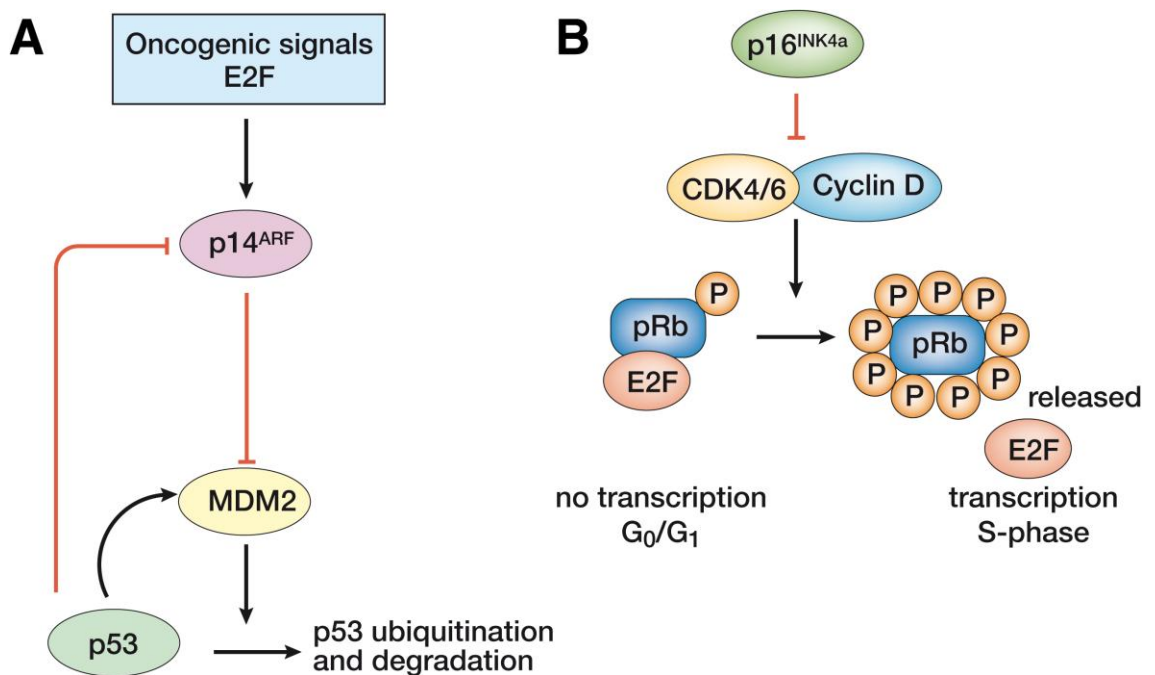
P27 null mice exhibit an overall increase in cell proliferation, suggesting that cells undergo additional cell divisions before differentiation, and they are more prone to spontaneous or induced tumorigenesis (Nakayama et al., 1996). In humans, it has been shown that abnormally low amounts of nuclear p27 are associated with poor clinical outcome and increased tumour aggressiveness in some tumours, for example breast and colon cancer (Loda et al., 1997) and non-small cell lung cancer (Esposito et al., 1997). P27 has a role in senescence, and it has been shown to be required for senescence in various cell settings and induced by various factors. For example, p27 is required for induction of Rb-mediated cellular senescence in human osteosarcoma cells (Alexander and Hinds, 2001), and for AKT1 induced senescence in mouse luminal epithelial cells (Majumder et al., 2008).

P27 also has some pro-tumorigenic functions, such as promoting cell cycle progression by facilitating assembly of cyclin D-CDK4 complexes when expressed at low levels (Cheng et al., 1999), and enhancing cell motility and thereby metastasis (Besson et al., 2004). Creation of a knock-in mouse model expressing a mutant p27 which cannot bind cyclins and CDKs has demonstrated p27's tumorigenic actions *in vivo*, as these mice develop spontaneous tumours caused by proliferation and tumour development from stem cells (Besson et al., 2007).

There is evidence suggesting that subcellular localisation of p27 can control its activity. It appears that nuclear p27 acts mainly as a tumour suppressor by inhibiting cyclin-CDK activity, and cytoplasmic p27 has oncogenic effects (Blagosklonny MV, 2002). This is supported by the finding that high nuclear levels combined with low cytoplasmic levels of p27 correlates with better prognosis in high-grade astrocytoma (Hidaka et al., 2009), whereas cytoplasmic localization of p27 correlates with high tumour grade and poor prognosis in several types of tumours (Slingerland and Pagano, 2000).

### 1.2.8 INK4A and INK4B locus

The region of human chromosome 9, where the *INK4b-ARF-INK4a* locus is located, which corresponds to regions on chromosome 4 in mice and chromosome 5 in rats, is one of the most frequently mutated sites in cancer (reviewed by (Ruas and Peters, 1998; Sharpless, 2005)). First *INK4a* was identified (Kamb et al., 1994; Nobori et al., 1994), which was followed by the identification of *INK4b* adjacent to *INK4a* (Hannon and Beach, 1994). *INK4a* encodes for two different splice variants, namely p16<sup>INK4a</sup> and p14<sup>ARF</sup>. P14<sup>ARF</sup> is translated from a different reading frame to p16<sup>INK4a</sup>, giving it its name ARF, which stands for Alternative Reading Frame (Quelle et al., 1995). The *INK4b* locus encodes p15<sup>INK4b</sup>, and both p16<sup>INK4a</sup> and p15<sup>INK4b</sup> act as CDKIs. p16<sup>INK4a</sup> and p14<sup>ARF</sup> both are involved in regulating cell cycle progression, but in different pathways. p16<sup>INK4a</sup> activates the pRb pathway by binding and inhibiting the action of cyclin D-CDK4/6 complexes, whereas p14<sup>ARF</sup> acts on the p53 pathway by counteracting MDM2-mediated degradation of p53 [(Bothner et al., 2001; Lowe and Sherr, 2003; Pomerantz et al., 1998); Figure 1.6, adapted from (Gil and Peters, 2006)]. Both transcripts are induced in response to stimuli such as oncogenic stress and aberrant growth, and both can be induced upon senescence. Evidence suggests that p16<sup>INK4a</sup> is more important in senescence and tumour suppression in human cells, whereas p14<sup>ARF</sup> seems more important in mouse cells. There is significant evidence that p16<sup>INK4a</sup> is important in preventing tumorigenesis, but there is less evidence for the importance of p14<sup>ARF</sup> in preventing tumorigenesis. This is because mutations that specifically affect p14<sup>ARF</sup> are rare, and p14<sup>ARF</sup> has not been studied in the context of cancer as much as p16<sup>INK4a</sup>. Studying the relative importance of *INK4a*, *ARF* and *INK4b* in tumour suppression is complicated by the fact that homozygous deletions in the region of chromosome 9 often affect all three genes. Most of the point mutations in the locus affect *INK4a*, rather than the *ARF* or *INK4b*, but there are also some types of tumour which are connected with specific inactivation of the *INK4b* or the *ARF* locus [reviewed by (Ruas and Peters, 1998; Sharpless, 2005)].



**Figure 1.6: p14<sup>ARF</sup> and p16<sup>INK4a</sup>**

p14<sup>ARF</sup> and p16<sup>INK4a</sup> are both involved in regulating cell cycle progression, but in different pathways. p14<sup>ARF</sup> acts on the p53 pathway by counteracting MDM2-mediated degradation of p53 (**A**), whereas p16<sup>INK4a</sup> activates the pRb pathway by binding and inhibiting the action of cyclinD-CDK4/6 complexes (**B**) [Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Gil and Peters, 2006), copyright (2006)].

### 1.2.9 P16<sup>INK4a</sup>

The first evidence pointing to p16<sup>INK4a</sup> as a tumour suppressor was the occurrence of missense mutations of p16<sup>INK4a</sup> in familial melanoma (Hussussian et al., 1994). It is now known that p16<sup>INK4a</sup> is inactivated in many tumours and tumour cell lines, by various mechanisms such as deletion, promoter methylation and point mutations. On the other hand, individuals which have a homozygous mutation which causes a severely truncated form of p16<sup>INK4a</sup> may not develop tumours for several decades, which suggests that homozygous loss of p16<sup>INK4a</sup> alone might not be sufficient to induce tumours, but it probably needs further mutations to induce tumorigenesis (Gruis et al., 1995a; Gruis et al., 1995b).

p16<sup>INK4a</sup> is up-regulated upon senescence and is maintained at high levels in senescent cells, suggesting it is important for induction and maintenance of senescence. In addition to up-regulation of p16<sup>INK4a</sup> in senescent cells, pRb is hypophosphorylated and E2F target genes are repressed (Alcorta et al., 1996; Hara et al., 1996; Stein et al., 1999). Reconstitution of telomerase activity in conjunction with p16<sup>INK4a</sup> inactivation immortalises some human cell types, for example breast epithelial cells (Kiyono et al., 1998).

P16<sup>INK4a</sup> can bind both monomeric Cdk4/6 and cyclinD-bound Cdk4/6. Binding to the monomeric Cdk4/6 subunit inhibits binding of cyclin D and causes the CDK to become non-activatable, and binding to the Cdk4/6–cyclinD complex leads to inhibition of the complex without dissociating it; in turn pRb phosphorylation by CDK4/CDK6 is prevented (Jeffrey et al., 2000). This seems to be the main function by which p16<sup>INK4a</sup> induces growth arrest, which is demonstrated by the finding that, in contrast to cells with normal pRb function, p16<sup>INK4a</sup> expression cannot efficiently arrest cell lines which lack pRb (Lukas et al., 1995).

p16<sup>INK4a</sup> transcription is induced in response to Ras signalling, via Ras–Raf–MEK kinase cascade activation of Ets-1, which in turn activates transcription of p16<sup>INK4a</sup> (Ohtani et al., 2001).

### 1.2.10 P14<sup>ARF</sup>

P14<sup>ARF</sup> (p19<sup>ARF</sup> in mice) was identified as a splice variant of the *INK4A* locus, and it has its own promoter and an alternative first exon, exon 1 $\beta$ , to p16<sup>INK4A</sup> (Quelle et al., 1995). It is translated from an alternate reading frame to p16<sup>INK4a</sup>, and therefore these two proteins share no amino acid homology. P14<sup>ARF</sup> exerts its anti-proliferative function by sequestering Mdm2 to the nucleolus, thereby inhibiting Mdm2-mediated ubiquitination and degradation of p53 [reviewed by (Gil and Peters, 2006)]. The N-terminal 25 amino acids are required for this activity, and this is encoded by the exon 1 $\beta$  (Quelle et al., 1995). P14<sup>ARF</sup> acts on Mdm2 following stress, making Mdm2 incapable of modifying other proteins [reviewed by (Sherr and Weber, 2000; Wesierska-Gadek and Schmid, 2005)]. It has been demonstrated that stabilization of p53 by p14<sup>ARF</sup> can be achieved without relocation of MDM2 to the nucleolus (Llanos et al., 2001). In addition to stabilizing p53 levels, p14<sup>ARF</sup> has also been shown to control cell proliferation in a p53-independent manner [reviewed by (Cleveland and Sherr, 2004)].

P14<sup>ARF</sup> seems to assume the more prominent role than p16<sup>INK4A</sup> in mice, where it was first indicated as a tumour suppressor as mice lacking the first exon of p19<sup>ARF</sup> were highly prone to spontaneous and carcinogen-induced tumours (Sharpless, 2005). The p19<sup>ARF</sup>-p53 pathway is the major senescence-inducing pathway in mice [reviewed by (Gil and Peters, 2006; Sharpless, 2005)]. In MEFs p19<sup>ARF</sup> is expressed upon senescence, and cells deficient of p19<sup>ARF</sup> do not enter senescence in culture and are transformed by Ras (Kamijo et al., 1997).

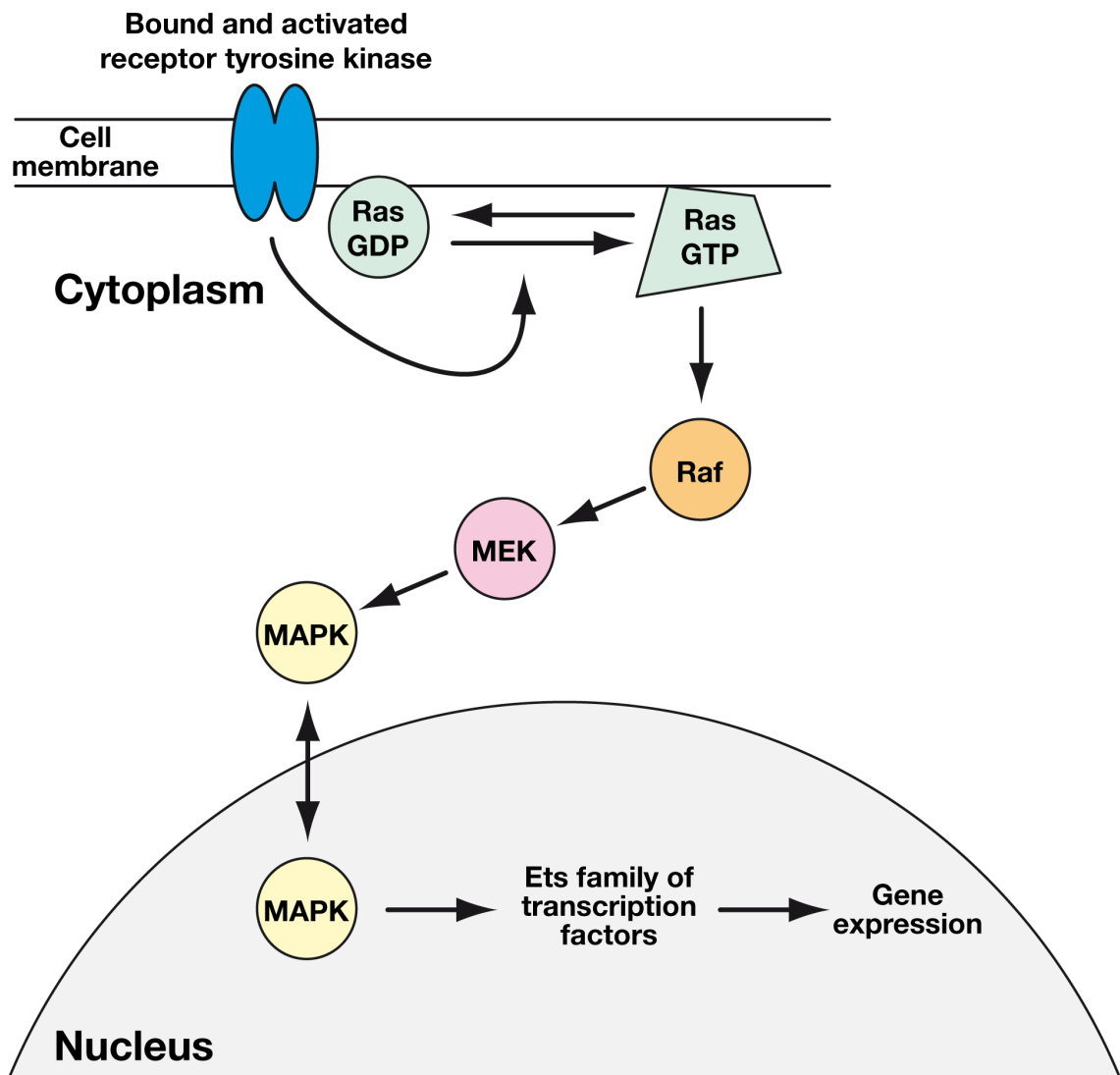
In humans, p14<sup>ARF</sup> specific mutations have been observed in familial melanoma-astrocytoma syndrome (Randerson-Moor et al., 2001). Furthermore, p14<sup>ARF</sup> promoter methylation has been reported in various cancers, for instance gliomas (He et al., 2010) and colon cancer (Burri et al., 2001). P14<sup>ARF</sup> over-expression has been shown to induce cell cycle arrest in human cells (Sekaric et al., 2007; Weber et al., 2002). But p14<sup>ARF</sup> does not seem to be critical for induction of senescence at least in some human cells, as demonstrated by the ability of human melanocytes to undergo oncogene-induced senescence in the absence of *INK4a/ARF*-encoded proteins (Haferkamp et al., 2009).

### 1.2.11 The Ras family

In 1964 it was demonstrated that preparations of a murine leukaemia virus taken from a leukaemic rat could induce sarcomas in new-born rodents (Harvey, 1964), and soon other viruses with oncogenic properties were identified (Kirsten and Mayer, 1967; Peters et al., 1974). It was found that the Harvey and Kirsten retrovirus strains were recombinant viruses which contained rat gene sequences (Scolnick et al., 1973). The rat sequences conveying the oncogenic properties were identified *Hras* in Harvey strain and *Kras* in the Kirsten retrovirus (Ellis et al., 1981). In the 1980s a human transforming gene was identified which was shown to be a member of the *RAS* family (Hall et al., 1983; Parada et al., 1982; Shimizu et al., 1983). The human *RAS* gene, *NRas*, was identified in human neuroblastoma and human sarcoma cell lines.

The protein products of the 3 human *ras* genes (*H-ras*, *N-ras* and *K-ras*) are GTP (guanosine triphosphate)/GDP (guanosine diphosphate; inactive form of GTP) activated switches (Barbacid, 1987; Boguski and McCormick, 1993; Bourne et al., 1990). Ras proteins are localized on the inner surface of the plasma membrane, and this localization is essential for their function. They transduce extracellular ligand-mediated stimuli into the cell, thereby controlling signal transduction pathways which are involved in cell proliferation, differentiation and apoptosis [reviewed by (Khosravi-Far and Der, 1994); Figure 1.7 adapted from (Campbell et al., 1998)]. Guanine nucleotide exchange factors (GEFs) promote formation of GTP-bound Ras (the active state), and Ras GTPase activating proteins (GAPs) promote formation of the inactive GDP-bound state of Ras (Boguski and McCormick, 1993; Quilliam et al., 1995). Mutations in the *Ras* genes have been identified in various types of tumour, such as colorectal cancer, bladder carcinoma, and in urinary tract tumours (Bos et al., 1987; Fujita et al., 1984; Visvanathan et al., 1988). Mutations found in these tumours always cause a constitutively active Ras protein, and it has been demonstrated that single point mutations can create a mutant Ras which cannot be inactivated by GAP, thereby producing a constitutively active Ras (Adari et al., 1988).





**Figure 1.7: Ras signal transduction**

Ras proteins are localized on the inner surface of the plasma membrane, and this localization is essential for their function. They transduce extracellular ligand-mediated stimuli into the cell, thereby controlling signal transduction pathways which are involved in cell proliferation, differentiation and apoptosis [Adapted by permission from Macmillan Publishers Ltd: [Oncogene] (Campbell et al., 1998), copyright (1998)].

Many signalling pathways converge at Ras, and Ras proteins are activated in response to many stimuli, such as growth factors, cytokines, neurotransmitters and hormones which stimulate cell surface receptors [reviewed by (Khosravi-Far and Der, 1994)]. Activated Ras transmits the signal by activating a cascade of cytoplasmic proteins, and the best characterized pathway is through Ras activation of Raf, the Ras–Raf–MEK kinase pathway (Figure 1.7). Ras interacts with Raf, recruiting Raf to the plasma membrane where Raf is activated [reviewed by (Marais et al., 1995; Morrison and Cutler, 1997)]. Signalling of active Raf activates mitogen-activated protein kinase (MAPK, also known as ERK) signalling, which in turn leads to activation of transcription factors, such as members of the Ets family (Wasylyk et al., 1998). These transcription factors control transcription of genes involved in proliferation and differentiation. Ras/Raf signalling induces cellular proliferation, but it also induces activation of CDKIs, such as p21 and p16<sup>INK4a</sup> [reviewed by (Lloyd, 1998)], which counteract proliferation.

Ras can induce cellular senescence, as first demonstrated by Newbold and Overell who found that oncogenic ras can transform most immortal rodent cell lines but it does not transform primary cells (Newbold and Overell, 1983). In primary cells oncogenic Ras results in a proliferative arrest shown to be cellular senescence (Franza et al., 1986; Hirakawa and Ruley, 1988; Ridley et al., 1988; Serrano et al., 1997). The initial effect of Ras on normal cells is to trigger proliferation, and following this aberrant proliferation cells enter cell cycle arrest. This arrest is induced by Ras/Raf induced expression of p16<sup>INK4A</sup> and p19<sup>ARF</sup>, which in turn activate Rb and p53 respectively [(Lin et al., 1998; Palmero et al., 1998; Zhu et al., 1998); reviewd by (Bringold and Serrano, 2000); Figure 1.8]. Ras/Raf signalling can also induce p21 independently of p53 activation (Sewing et al., 1997; Woods et al., 1997).

It was shown that Ras can cooperate with certain other oncogenic alterations to transform primary rodent cells (Land et al., 1983; Ruley, 1983). In primary murine fibroblasts lacking either p53 or p16<sup>INK4a</sup> transformation can be achieved by oncogenic Ras alone (Serrano et al., 1996; Tanaka et al., 1994).

**Figure 1.8: Oncogene-induced senescence** (*Figure has been removed due to Copyright restrictions.*)

The initial effect of Ras on normal cells is to trigger proliferation, and following this aberrant proliferation cells enter cell cycle arrest. This arrest is induced by Ras/Raf induced expression of p16<sup>INK4A</sup> and p14<sup>ARF</sup>, which in turn activate Rb and p53 respectively. Ras/Raf signalling can also induce p21 independently of p53 activation [figure adapted from (Bringold and Serrano, 2000)].

### **1.3 DNA tumour viral proteins that de-regulate the cell cycle**

DNA tumour viruses' natural hosts are differentiated cells. As differentiated cells do not proliferate, the viruses' lytic cycle is dependent on their ability to control the replicative machinery of their host. Therefore, like tumour cells, DNA tumour viruses evolved mitogenic mechanisms that overcome the intracellular and extracellular factors that normally control cellular replication. These viruses can alter transcription to promote the expression of proteins required for viral replication, and overcome the finite proliferative potential of cells. Many of the cell cycle checkpoints that DNA viruses deregulate are also deregulated in tumour cells. For instance, some DNA viruses can inactivate the pRb pathway or the pRb and p53 pathways, which are frequently inactivated in tumour cells. Viral proteins such as Simian Virus 40 large tumour antigen (SV40 LT), Human papillomavirus 16 E7 (HPV Type 16 E7) and Adenovirus Type 5 E1A all function as potent viral oncoproteins to induce immortalisation and transformation of many cell types (Braithwaite et al., 1983; Caporossi and Bacchetti, 1990; Chang et al., 1997; Duensing and Munger, 2002).

DNA tumour viruses are a powerful tool for studying signalling pathways and have contributed strongly to our basic understanding of the cellular pathways that drive tumorigenesis. Before the use of recombinant DNA technologies DNA tumour viruses were used extensively to study cancer, and most of the beginnings of the field of cancer biology reside in the study of viral mediated transformation. The importance of the pRb and p53 pathways in tumorigenesis was first revealed with the help of DNA tumour viruses.

#### **1.3.1 Simian Virus 40 large tumour antigen**

SV40 belongs to the family of *Polyomaviridae*, which are small icosahedral viruses. The tumorigenic activity of SV40 was first demonstrated by its ability to transform several hamster and rodent cell lines, and by the fact that infection with SV40 caused the formation of tumours in hamsters [reviewed in (Hilleman, 1998)]. The SV40 genome encodes three structural components of the virion (VP1, VP2, and VP3), as well as the three non-structural proteins large T (LT) and small t antigen, and 17kT

antigen. If SV40 infects permissive cells it enters its normal lytic cycle, leading to host cell lysis and the production of infectious progeny virions. But if SV40 infects non-permissive cells, such as rodent cells, host cell lysis and progeny virion production does not occur. Instead, infected cells are forced to enter S-phase. Failure to induce the normal lytic cycle seems to be caused by the fact that in non-permissive cells SV40 fails to initiate viral DNA replication and activate transcription from the late promoter, but early events including production of T antigens occurs normally (Ahuja et al., 2005).

The LT protein alone is sufficient for many of the functions required for the SV40 life cycle. Also, its expression is necessary and often sufficient to immortalize many cell types. For example, LT is sufficient to bypass replicative senescence of rat embryo fibroblasts, but if LT is inactivated in these cells, they rapidly enter irreversible arrest in G<sub>1</sub> or G<sub>2</sub>, demonstrating the requirement of LT to remain immortalized (Jat and Sharp, 1989). Furthermore, this shows that the cell cycle control and checkpoints remain intact during immortalization of REFs with LT. REFs only become dependent on LT once their normal proliferative potential has been used up. The LT transforming function can be explained by its ability to impair the activity of two host cell proteins involved in anti-tumorigenesis, p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) and pRb (Decaprio et al., 1988). It can also impair the activity of many other host cell proteins, such as p107 (Dyson et al., 1989b; Ewen et al., 1989), p130 (Hannon et al., 1993), TBP (Martin et al., 1993), BUB1 (Cotsiki et al., 2004) and CBP and p300 (Avantaggiati et al., 1996; Eckner et al., 1996).

LT binds to Rb family members via its LXCXE-binding domain (Decaprio et al., 1988; Moran, 1988). It binds to and sequesters the active, hypophosphorylated form of Rb (Ludlow et al., 1989; Ludlow et al., 1990), thereby promoting release of E2F which in turn activates transcription of E2F responsive genes. Rb binding of LT is required for its ability to immortalize cells, as mutants which are defective for Rb binding have a reduced ability to immortalize (Decaprio et al., 1988; Powell et al., 1999). Rb binding also seems to be required for LT's ability to transform cells as shown by the fact that some pRb-binding LT mutants are defective for transformation (Chao et al., 2000).

LT also binds to p53, and p53 was discovered as a cellular protein which binds to LT in SV40-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). LT

inhibits p53 from binding to promoters and regulating gene expression by interacting with its DNA binding surface (Bargonetti et al., 1992; Jiang et al., 1993). It has been shown that the C-terminal domain of LT is sufficient to inhibit p53 activity (Cavender et al., 1995), and that p53 binding occurs via a bipartite region in the C-terminal domain. LT can inhibit p53-dependent transcription and growth arrest not only by p53 binding but also independently of p53 binding (Rushton et al., 1997). SV40 transformed cells have large amounts of p53 because binding of LT to p53 results in stabilization of p53, but the p53 is functionally inactive (Oren et al., 1981). This stabilization of p53 might be caused by the association of p300, which is involved in Mdm2-mediated p53 degradation (Grossman et al., 1998), and Mdm2 with p53 when bound to LT (Brown et al., 1993; Henning et al., 1997).

### **1.3.2 Adenovirus type 5 E1A**

Adenovirus type 5 is a double stranded DNA virus which infects epithelial cells in the respiratory tract. The protein products encoded by genes in the early region 1A (E1A) cause viral transcription and re-programme cellular gene expression to overcome cell cycle arrest. Primary rodent cells can be immortalized by expression of E1A (Houweling et al., 1980). If E1A is expressed in quiescent rodent cells it also causes them to re-enter the cell cycle [reviewed by (Bayley and Mymryk, 1994; Gallimore and Turnell, 2001)], but it can also induce apoptosis in growth-arrested rodent cells (Mymryk et al., 1994).

The first E1A binding protein identified was pRb (Whyte et al., 1988), and E1A binds pRb mainly through an LXCXE motif within CR2. This interaction disrupts pRb-E2F complexes and enables E2F to promote entry into S phase (Sherr, 1996). E1A also binds the other members of the pRb family p107 and p130 (Whyte et al., 1988). E1A pREFerentially, but not exclusively, binds to the active hypophosphorylated pRb (Mittnacht et al., 1994).

If E1A is overexpressed at high levels it can induce apoptosis (Mymryk et al., 1994; Rao et al., 1992). One mechanism by which E1A can induce apoptosis is via induction of p14<sup>ARF</sup> by E2F which inhibits Mdm2-dependent p53 degradation, thereby allowing

p53 to accumulate (de Stanchina et al., 1998). In many tumour cell lines stable expression of E1A does not induce apoptosis but revert the transformed phenotype, which was first demonstrated by Frisch (Frisch, 1991; Frisch and Dolter, 1995).

E1A enhances acetylation of pRb by p300, and acetylation of pRb by p300 blocks phosphorylation of pRb by cyclin E-CDK2 (Chan et al., 2001). Acetylation of pRb promotes it to interact with Mdm2 (Chan et al., 2001), and pRb, p53 and Mdm2 form complexes. It has been shown that such trimeric complexes prevent degradation of p53 and allow p53-mediated transcriptional repression and p53 mediated induction of apoptosis, while inhibiting p53-mediated transcriptional activation of target promoters (Hsieh et al., 1999) thereby dissociating the apoptotic function of wild type p53 from its transactivation function [reviewed by (Yap et al., 1999)]. Thereby E1A can stabilize p53 and increase the host cells sensitivity to apoptotic signals, which has been observed in many cells (Lowe and Ruley, 1993).

### **1.3.3 Human papillomavirus 16 E7**

HPV is a non-enveloped double-stranded DNA virus with an icosahedral capsid which infects mucosal and cutaneous epithelial cells [reviewed by (Longworth and Laimins, 2004)]. Approximately 118 types of HPV have been identified (Zandi et al., 2010), which can be grouped into low risk, which cause benign warts and lesions, and high risk which cause malignant transformation [reviewed by (Cutts et al., 2007)]; type 16 and type 18 are high risk. The viral genome codes for 6 early genes, E1 – E7, and two late genes, L1 and L2. During the normal life cycle the virus replicates extrachromosomally, but when the virus integrates into the host cells genome the oncoproteins that cause transformation, E6 and E7, are over-expressed.

The HPV-16 E7 oncogene is approximately 100 amino acids long (Munger and Howley, 2002), and it binds to and inactivates pRb, leading to release of E2F which can in turn activate transcription from E2F responsive promoters (Dyson et al., 1989a). E7 binds to the active hypophosphorylated form of pRb, and it binds pRb with its LXCXE motif (Liu et al., 2006). In cells overexpressing E7 the G<sub>1</sub>/S phase checkpoint is lost, causing aberrant cellular proliferation (Dyson et al., 1989a; Dyson, 1998). In addition to

inactivating pRb, it has also been demonstrated that E7 can cause ubiquitin-proteasome mediated degradation of pRb (Boyer et al., 1996). E7 also binds to other proteins such as transcriptional co-repressor proteins HDACs (Brehm et al., 1999), which are proteins recruited by pRb to actively repress E2F transcription [reviewed by (Frolov and Dyson, 2004)]. Furthermore it has been shown that E7 can bind to and activate cyclin/CDK2 complexes, which in turn phosphorylate and inactivate pRb and induce transcription of genes required for S phase (Arroyo et al., 1993; McIntyre et al., 1996). It has also been demonstrated that E7 can cause aberrant proliferation by binding to and inhibiting the CDKIs p21 and p27 (Funk et al., 1997; Zerfass-Thome et al., 1996).

HPV-16 is a high risk HPV type which causes malignant transformation (Cutts et al., 2007). Holland and colleagues found that HPV-16 E7 can induce transcription of enhancer of zeste homologue 2 (EZH2) through E2F transcription factors, which in turn enhances proliferation of cells by bypassing the G<sub>1</sub>/S checkpoint and inhibits apoptosis (Holland et al., 2008). Baldwin and colleagues performed a screen for kinases targeted by HPV-16 E7 and identified 5 such kinases essential for proliferation and cell survival, namely CDK6, FYN, adaptor protein 2 associated kinase 1 (AAK1), testis-specific serine/threonine kinase 2 (TSSK2) and epidermal growth factor receptor-related protein tyrosine kinase B3 (ERBB3) (Baldwin et al., 2008).

HPV-16 E7 can also lead to increased levels of expression of interleukin-6 (IL-6) and the anti-apoptotic protein Mcl-1 (Cheng et al., 2008). It has also been shown that E7 can bind to and inactivate interferon regulatory factor 1 (IRF-1) (Park et al., 2000; Um et al., 2002), thereby contributing to the immune evasion of HPV-infected tumour cells. In addition to being a key regulator of the cellular immune response, IRF-1 has also been shown to act as a negative regulator of proliferation [reviewed by (Romeo et al., 2002)], therefore inhibition of IRF-1 by E7 could contribute towards the aberrant proliferation induced by E7.

The HPV E6 protein consist of approximately 150 amino acid polypeptides , and they have four Cys-X-X-Cys motifs which can form two zinc fingers [reviewed by (Thomas et al., 1999)]. The HPV-16 and HPV-18 E6 proteins are oncogenes which can bind to and destabilise p53 by causing ubiquitin induced degradation of p53 (Scheffner et al., 1990). E6 has also been demonstrated to affect telomerase activity; Klingelhutz and



colleagues (Klingelhutz et al., 1996) found that HPV-16 E6 activates telomerase in early-passage human breast epithelial cells and keratinocytes. Telomerase was activated before cells entered crisis and became immortal. Functions of E6 extended the lifespan, but E6 introduction was insufficient to immortalize cells.

#### **1.4 Senescence, crisis and immortalisation**

As first described by Hayflick (Hayflick and Moorhead, 1961), normal cultured somatic cells have a limited proliferative potential, and once they have used up this potential they enter senescence, also known as mortality 1 stage, M1. If cells are transformed with viruses which can inhibit pRb and p53 function, their proliferative potential can be extended (Lustig, 1999). Following this extended period of proliferation, cells reach crisis, also termed M2 (mortality stage 2) [reviewed by (Lustig, 1999)] which is defined by ‘uncapped’ chromosome ends, end-fusions, chromosome breakage and fusion, mitotic catastrophe and a high proportion of apoptotic cells [reviewed by (Shay and Wright, 2005)]. Crisis occurs because cells continue to divide in spite of their telomeres becoming critically short (Lustig, 1999; Shay and Wright, 2005). During crisis an increase in cell death rate and chromosomal abnormalities occurs. Both, M1 and M2, represent potential anti-tumour mechanisms. At a low frequency, cells can survive crisis and become spontaneously immortalised; this is believed to occur due to activation of endogenous telomerase activity or ALT (Shay and Wright, 2005).

Bodnar and colleagues have demonstrated that introduction of hTERT into two telomerase-negative normal human cell types, retinal pigment epithelial cells and BJ foreskin fibroblasts was sufficient to immortalise these cells (Bodnar et al., 1998). Further studies seemed to demonstrate that introduction of hTERT was sufficient to immortalize cells (Ouellette et al., 2000; Vaziri and Benchimol, 1998; Yang et al., 1999). In contrast, others have found that activation of telomerase is not enough to immortalize cells (Counter et al., 1998; Hahn et al., 1999; Kiyono et al., 1998). Kiyono and colleagues (Kiyono et al., 1998) found that inactivation of the pRB/p16<sup>INK4</sup> pathway was required in addition to telomerase activation to immortalize neonatal keratinocytes and adult mammary epithelial cells. Counter and colleagues immortalized human

fibroblasts and embryonic kidney cells by introducing SV40 LT antigen in addition to hTERT (Counter et al., 1998; Hahn et al., 1999). It has been suggested that when cells are cultured under adequate conditions hTERT is sufficient to immortalize; Herbert and colleagues found that primary HMECs (human mammary epithelial cells) can be immortalized by introduction of hTERT without abrogating the pRb/p16<sup>INK4</sup> pathway when cells were grown on feeder layers (Herbert et al., 2002).

Mike O'Hare, Parmjit Jat and colleagues (O'Hare et al., 2001) found that hTERT alone or a temperature-sensitive (ts) mutant (U19tsA58) of SV40 LT antigen alone (or normal SV40 LT alone) were not sufficient to immortalize freshly isolated normal adult human mammary fibroblasts and endothelial cells. However, combined expression of hTERT and ts SV40 LT yielded immortal cell lines. Immortalization occurred irrespective of the order in which these factors were introduced, and irrespective of whether they were introduced early or late in the normal proliferative lifespan of these cultures. The temperature sensitive LT antigen becomes inactive upon shift to a higher temperature, the non-permissive temperature, and when the immortalized cultures were shifted to this temperature cultures arrested, demonstrating that maintenance of the immortalized state depended on continued expression of functional LT antigen, with hTERT alone insufficient to maintain growth. Within 7 days of incubation at the non-permissive temperature cultures arrested irreversibly, even though telomeres had been lengthened and telomerase was still active.

## **1.5 Development of conditionally immortalized human breast luminal epithelial cells**

Studying cellular senescence is complicated by the fact that it occurs asynchronously in heterogeneous cell populations. To overcome this problem, model systems of oncogene-induced senescence, stress-induced senescence and irradiation-induced senescence, which can be induced prematurely and acutely, have been studied. Mike O'Hare, Parmjit Jat and colleagues (O'Hare et al., 2001) used another approach. The requirement of hTERT and SV40 LT in combination to immortalise cells enabled them to develop conditionally immortalized human mammary fibroblasts to study senescence, by using

hTERT in conjunction with the ts mutant SV40 LT, U19tsA58 (O'Hare et al., 2001). These cultures are conditional for growth as inactivation of the LT antigen results in a rapid growth arrest; the cells proliferate at 34°C but undergo a synchronous growth arrest upon shift to 38°C. O'Hare and colleagues used the same approach to conditionally immortalize freshly isolated human breast luminal epithelial and myoepithelial cells (unpublished data summarised in this thesis).

### **1.5.1 226L8/13 cells**

226L 8/13 are luminal epithelial cells immortalised with hTERT and ts SV40 LT antigen. These cells were derived from a fresh reduction mammoplasty HBr 229. The epithelial cells were immunomagnetically sorted to prepare an enriched culture of luminal epithelial cells. HBr 229 were grown on 3T6\* feeder cells, and when the cells were dividing well they were transduced with amphotropic viral supernatants in E93 medium overnight in the presence of 8 µg/ml polybrene. They were transduced with the constructs previously used by O'Hare and colleagues (O'Hare et al., 2001) to immortalise freshly isolated normal adult human mammary fibroblasts and endothelial cells, namely pBabehygrohTERT Clone13 expressing hTERT (the catalytic subunit of human telomerase), and pZIPNeoU19tsA58 Clone 8 expressing U19tsA58 LT (the temperature sensitive SV40 LT). Following transduction cells were selected for G418 and hygromycin resistance. The transduced cells were then serially single cell cloned to deplete their finite proliferative potential. Luminal cells underwent very few population doublings even upon introduction of hTERT. However, SV40 LT extended the lifespan of these cells extensively. Because SV40 LT extended the lifespan extensively, it was necessary to serially single cell clone cells transduced with hTERT and SV40 LT to deplete the extended life span. It took seven rounds of serial single cell cloning before SV40 LT transduced cells stopped dividing; Cells transduced with hTERT and SV40 LT continued dividing even after seven rounds of single cell cloning. The single cell cloning was performed in low oxygen conditions and using a 3T6\* feeder layer. Cultures were negatively sorted against the myoepithelial markers CALLA (Common acute lymphocytic leukemia antigen) and  $\beta$ -4 integrin to remove myoepithelial cells at

each round of single cell cloning. This was repeated until the 226L 8/13 cells had reached clonal level eight.

### **1.5.2 Cell types of the human breast**

The breast stroma consists of many cell types, which include fibroblasts, adipocytes, endothelial cells and inflammatory cells. Myoepithelial cells and luminal epithelial cells make up the mammary gland ductal system. Luminal epithelial cells line the lumen of the mammary gland, and there are two types, ductal and alveolar cells. Myoepithelial cells are contractile cells that surround the luminal cells and are in contact with the basement membrane. With each menstrual cycle and during pregnancy the mammary gland undergoes proliferation. It is believed that stem cells are present at the tip of these ducts (mammary stem cells, MaSCs) which give rise to luminal epithelial and myoepithelial cells during these proliferative cycles [reviewed by (Visvader, 2009)].

There is evidence that the mammary epithelium is organized in a hierarchical manner [reviewed by (Visvader, 2009)]. This developmental process begins with an undifferentiated oestrogen receptor–negative MaSC which self-renews to maintain its pool and can differentiate into committed common progenitors. It is not clear if there are one or more types of progenitor. These common progenitors differentiate into the luminal progenitors which give rise to the mature luminal epithelial cell lineage, both ductal and alveolar cells, and the myoepithelial progenitors which give rise to mature myoepithelial cells. Alveolar progenitor cells give rise to alveolar cells, but they might also be able to differentiate into myoepithelial progenitors (Figure 1.9).

**Figure 1.9: Breast cells and their progenitors** (*Figure has been removed due to Copyright restrictions.*)  
Schematic model of the differentiation hierarchy of the mammary epithelium [figure adapted from (Visvader, 2009)].

It is not clear from which cell type breast cancer usually originates. Human breast cancer can be categorized into subtypes that have properties consistent with both basal and luminal origin. Breast cancer is a heterogeneous disease, and the presence of six molecular subtypes, namely the luminal A and luminal B subtypes (both oestrogen receptor alpha-positive), the normal-like, HER2-positive, basal subtypes (comprising triple-negative cancers), and the claudin-low subtype [reviewed by (Yalcin-Ozuysal and Briskin, 2009)]. This heterogeneity of breast cancers could be explained by two hypotheses; the cell of origin hypothesis proposes that breast cancers can originate in different cell types, including breast epithelial stem and progenitor cells, transit amplifying cells and differentiated cells [reviewed by (Stingl and Caldas, 2007)]. The cancer stem cell (CSC) theory proposes that solid breast tumours are hierarchically organized, and that a subpopulation CSCs self-renew and give rise to different cell types, thereby sustaining the tumour [reviewed by (Visvader, 2009)].

The most common subtypes of breast cancer are luminal subtypes (Petersen et al., 2003), but cancers that express myoepithelial (basal) markers have a poorer prognosis (El Rehim et al., 2004). In the Carolina Breast Cancer Study, luminal breast cancers made up 67% of the tumours analysed [reviewed by (Brenton et al., 2005)]. Generally, luminal subtypes have a good prognosis, but luminal B carries a significantly worse prognosis than luminal A [(Sorlie et al., 2003), reviewed by (Brenton et al., 2005)]. The luminal subtypes are hormone receptor-positive breast cancers and have expression patterns reminiscent of luminal epithelial cells, which include expression of luminal cytokeratins 8/18, ER and genes linked with ER activation like LIV1 and CCND1 [(Perou et al., 2000; Sotiriou et al., 2003), reviewed by (Brenton et al., 2005)].

## **1.6 shRNA libraries and studying gene function**

RNA interference (RNAi) was first discovered by Fire and colleagues, who demonstrated the ability of double-stranded RNA homologous to a specific gene to silence this gene's expression in the nematode worm *Caenorhabditis elegans* (Fire et al., 1998). A problem that needed to be overcome before RNAi could be used in mammalian cells was the induction of innate immune pathways and shutdown of

cellular protein expression by mammalian cells in reaction to long dsRNA, which is part of the cells anti-viral mechanism. If dsRNAs longer than 29–30 bp are introduced into mammalian cells the innate immune system is induced via dsRNA dependent protein kinase (PKR) (Williams, 1997). This was overcome when it was demonstrated that introduction of synthetic small interfering RNA (siRNA), which are shorter and therefore do not trigger PKR activation, could achieve sequence-specific knock-down of genes in mammalian cells (Elbashir et al., 2001a). Such siRNAs can be introduced into target cells using several standard transfection systems. RNAi occurs in plant and animal cells, and it is a sequence specific post-transcriptional mechanism of gene-silencing, which is initiated by double-stranded RNA (dsRNA) with a homologous sequence to the silenced gene [reviewed by (Sharp, 2001; Tuschl, 2001)]. RNAi provides a fast and convenient method to study gene function in mammalian cells, as it can achieve efficient knock-down of single or multiple genes [reviewed by (Bantounas et al., 2004)].

One theory for the evolution of RNAi is that it primarily evolved as a protective mechanism against RNA viruses or transposable elements [reviewed by (Waterhouse et al., 2001)]. It is also possible that RNAi evolved as a mechanism to negatively regulate expression of endogenous genes. MiRNAs are endogenous dsRNAs that negatively regulate gene expression through the RNAi pathway [reviewed by (Bartel, 2004; He and Hannon, 2004)]. In release 16 of the Sanger Institute miRbase 15172 miRNAs are annotated [<http://www.mirbase.org/>]; (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011)], a number which is constantly rising (e.g. 922 annotated miRNAs were in version 14). Many putative target genes have been identified, and different methods are used to detect miRNA targets, such as computational prediction, genetic approaches, miRNA overexpression or knockdown followed by microarray analysis [reviewed by (Thomas et al., 2010)]. Typically, miRNAs pair imperfectly with the 3'-untranslated region (UTR) of their cognate mRNAs, making identification of targets more complicated. It is believed that the degree of mismatch with the target mRNA sequence determines whether the target mRNA is degraded or translation of the target mRNA is inhibited. Pairing between the 'seed region' of an shRNA (nucleotides 2-8 on the 5' end of the guide strand) and complementary sequences in the 3'UTR of the target mRNA

determines specificity [reviewed by (Naito et al., 2009a; Naito et al., 2009b)]. MicroRNAs are involved in the regulation of many functions, such as developmental timing, differentiation, proliferation, antiviral defence and metabolism (Thomas et al., 2010).

### **1.6.1 Mechanism of RNA interference**

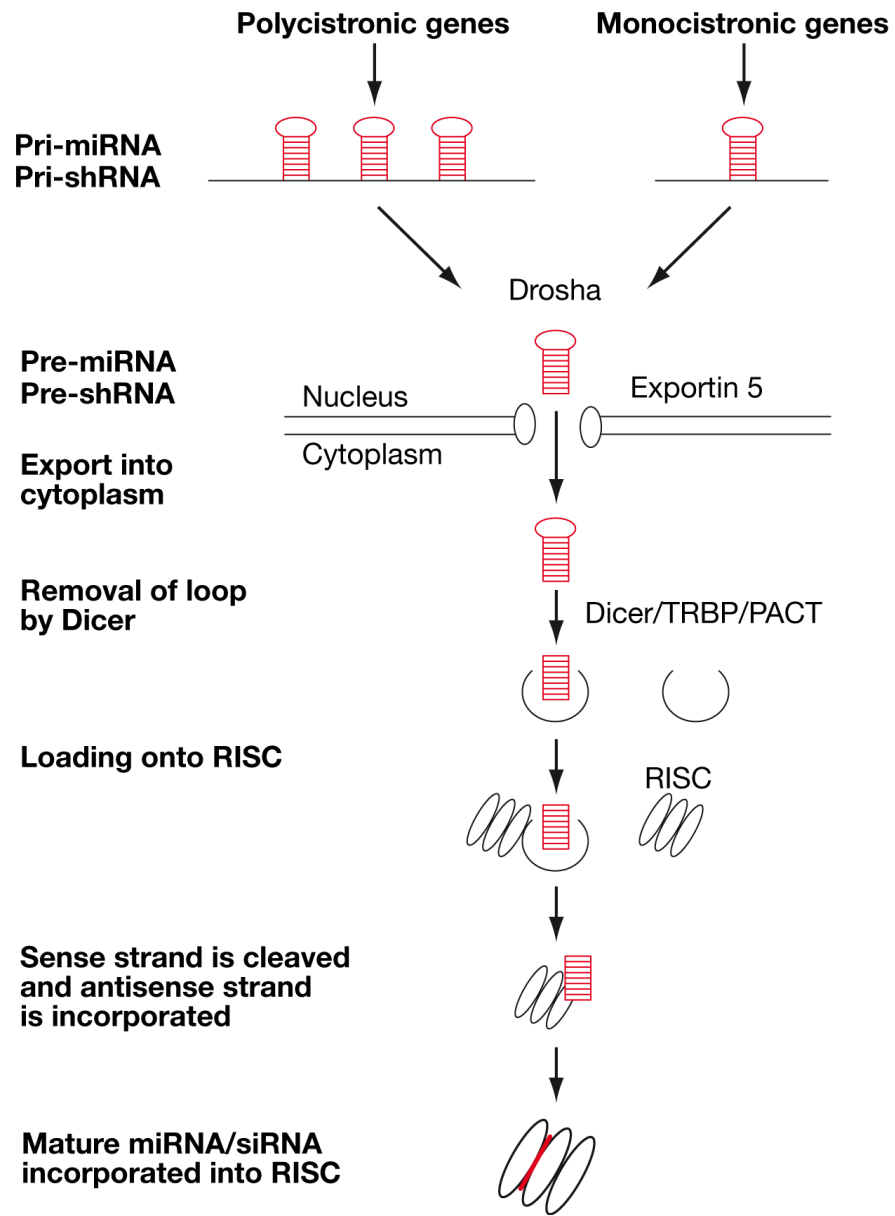
shRNAs (short hairpin RNAs) are non-coding dsRNAs which can be processed by cells into siRNAs, and their design is based on knowledge about miRNA synthesis, function and maturation [reviewed by (Pekarik, 2005)], especially the miR-30 based shRNAs (Silva et al., 2005). Early shRNAs had stems of 19-29 nucleotides and were expressed using RNA polymerase III promoters. Later on shRNAs were developed which imitate endogenous miRNAs, which are under the control of RNA polymerase II promoters and expressed as long 5'-capped and polyadenylated primary shRNAs (Naito et al., 2009b).

shRNAs can be transcribed by RNA polymerase II or III, and the primary transcript contains a hairpin and stem and loop which can then be processed to generate siRNAs. The long primary transcripts, the pri-shRNAs (equivalent to primary miRNAs, microRNAs), are processed into shRNAs with 2 nucleotide 3' overhangs by a complex which contains the RNase III enzyme Drosha transcript, known as pre-shRNAs (equivalent to pre-miRNAs) [(Lee et al., 2003; Zeng et al., 2005); reviewed by (Pekarik, 2005)]. Exportin 5 transports the pre-shRNA into the cytoplasm (Yi et al., 2003), where the loop of the hairpin is removed by a complex containing the RNase III related enzyme Dicer and TRBP/PACT, resulting in a double stranded siRNA (mature miRNA) of approximately 22 nucleotides with a 2 nucleotide 3' overhang (Bernstein et al., 2001; Elbashir et al., 2001b; Zhang et al., 2002). SiRNAs are then loaded onto the RNA-induced silencing complex (RISC), which contains members of the Argonaute (Ago) protein family (Hammond et al., 2001; Nykanen et al., 2001; Pham and Sontheimer, 2004). Which strand is incorporated into the RISC complex seems to be dependent on the sequence composition at the ends of the siRNA duplex; usually the strand whose 5' terminus is at the thermodynamically less stable end of the duplex gets incorporated into RISC (Khvorova et al., 2003a; Schwarz et al., 2003; Tomari et al., 2004). Helicase activity in the RISC complex unwinds the siRNA and the sequence of the incorporated



single-stranded RNA directs the complex to homologous mRNA sequences. The endogenous target RNA is then cleaved by RISC endonuclease activity where the sequence is homologous to the siRNA sequence (Elbashir et al., 2001b; Meister et al., 2004; Rivas et al., 2005). The activated RISC complex can carry out multiple rounds of RNA cleavage (Hutvagner and Zamore, 2002). Depending on mismatch between the target sequence and the siRNA sequence, the mRNA may not be degraded but rather translation inhibited [reviewed by (Pekarik, 2005)]. miRNA and shRNA biogenesis is illustrated in Figure 1.10.

Microarray profiling has demonstrated that off-target effects, i.e. unintended gene silencing, occur frequently because of sequence similarity between such off-target mRNAs and the seed sequence of the siRNA (nucleotides 2 to 8 on the 5' end of the guide strand) [reviewed by (Naito et al., 2009b)]. Ui-Tei and colleagues showed a correlation between the thermodynamic stability [or calculated melting temperature ( $T_m$ )] for the formation of the protein-free seed-target duplex, and the ability of an siRNA to cause off-target effects (Ui-Tei et al., 2008). Naito and colleagues showed that shRNAs with a low  $T_m$  in the seed-target duplex had little or no off-target effects, whereas with shRNAs with a high  $T_m$  in the seed-target duplex high levels of off-target effects were observed (Naito et al., 2009b). These results suggest that shRNAs with a low probability of having off-target effects could be constructed.



**Figure 1.10: miRNA and shRNA biogenesis**

Schematic diagram of the steps involved in the maturation of miRNAs/shRNAs

### **1.6.2 shRNA library screens**

Several different methods have been used to down-regulate protein levels, such as GSEs (genetic suppressor elements) and ribozymes which cause degradation of specific RNAs. But these methods are not very efficient in complex mammalian systems, and they are limited in their use in genome-wide screens. RNAi represents a method to down-regulate gene expression which can be used on the cell or organismal scale, and due to its relative simplicity and genome-wide availability, in a high throughput fashion and for genetic loss of function screens.

Elbashir and colleagues (Elbashir et al., 2001a) demonstrated that introduction of synthetic small interfering RNA (siRNA) could achieve sequence-specific knock-down of genes in mammalian cells. Such siRNAs can be introduced into target cells following several standard transfection systems, and they can be used for high throughput screens, but these chemically synthesized siRNAs are very expensive to synthesize. How strong the knock-down is depends on transfection efficiency, i.e. amount of siRNA introduced into cells, and the knock-down efficacy of the individual siRNAs (Hannon and Rossi, 2004).

Following the discovery of miRNAs in mammalian cells, shRNAs were developed based on the knowledge about miRNAs (Pekarik, 2005). SiRNAs have relatively short half lives, and to increase the length of expression of siRNAs expression, vectors were developed which express shRNAs from eukaryotic RNA polymerase II and III promoters (Chang et al., 2006). These expression vectors can be introduced into cells by standard transfection protocols, or they can be packaged into viruses, for instance retroviruses and lentiviruses, and then stably transduced into cells. Expression of such shRNAs can be either constitutive or inducible. shRNA libraries which cover the whole genome have been developed, which can be used in loss of function genetic screens (Chang et al., 2006; Root et al., 2006; Silva et al., 2005). Pooled screens have been successfully performed by several groups, for example screens revealing genes whose expression is required by cancer cells for proliferation and survival (Schlabach et al., 2008; Silva et al., 2008), or genes whose silencing selectively impairs the viability of Ras mutant cells (Luo et al., 2009).

## 1.7 Aims of the research

Studying cellular senescence in serially sub-cultivated cells is difficult due to the asynchrony of senescence growth arrest. Therefore, a conditionally immortalised human mammary fibroblast cell line had been developed in the Jat laboratory by retroviral transduction of early passage, adult interlobular mammary fibroblasts with a temperature sensitive non-DNA-binding mutant SV40 LT (U19tsA58) and hTERT, namely HMF3A cells (O'Hare et al., 2001). The HMF3A conditional growth arrest is critically dependent upon inactivation of LT since, at the permissive temperature, 34°C HMF3A cells grow normally whereas, at the non-permissive temperature, 38°C, HMF3A cells enter into an irreversible state of growth arrest within a period of between 5-7 days that is phenotypically indistinguishable from cellular senescence. The HMF3A cells have been used to study cellular senescence by microarrays, RNAi and *in silico* promoter analysis to promote the dissection of the signalling pathways responsible for regulating cellular senescence (Hardy et al., 2005; Rovillain et al., 2011).

Cellular context is highly significant in the development of cancer, and most cancers originate in epithelial cells. Considering the importance of cellular context and that most cancers originate in epithelial cells, conditionally immortalized human breast epithelial cells were developed, named 226L 8/13, which were derived from human breast luminal epithelial cells. These cells represent a highly relevant model to study cellular senescence. 226L 8/13 are epithelial counterparts of the HMF3A fibroblasts, and were immortalized by introduction of U19tsA58 and hTERT.

The aims of this thesis were:

1. To characterise of 226L 8/13 cells in terms of expression of cell type specific markers of epithelial, luminal epithelial and myoepithelial cells. Senescence has mainly been studied in fibroblasts, but most cancers arise in epithelial cells. Therefore, epithelial cells are a highly relevant model to study senescence.
2. To characterise the growth of 226L 8/13 cells at the permissive temperature, i.e. the temperature at which ts LT is active, and the non-permissive temperature, i.e.

the temperature at which ts LT is inactive. To establish if 226L 8/13 cells arrest at the non-permissive temperature, and if this arrest exhibits features of cellular senescence.

3. To optimise a growth complementation assay with the conditional cells and determine the importance of the p53 and pRb pathways in mediating the conditional growth arrest of 226L 8/13 cells.
4. To use the complementation assay to perform a loss of function shRNA screen in the conditional system to identify mediators of cellular senescence in the 226L 8/13 cells which might represent mediators of cellular senescence in human breast epithelial cells.

## **2 Materials and Methods**

### **2.1 Mammalian cell culture**

#### **2.1.1 Cell lines**

Φ amphotropic, Φ ecotropic and Human Embryonic Kidney 293 T/17 cell lines (HEK 293 T/17) were obtained from the ATCC. 226L 8/13, EcoR Clone 7 and HMF3A cells were made by Mike O'Hare and Parmjit Jat (O'Hare et al., 2001).

#### **2.1.2 Cell media**

226L 8/13 cells were cultured in E93: Dulbecco's Modified Eagle Medium/ Ham's F12 (DMEM/F12; 1:1; Invitrogen), supplemented with 2 millimolar (mM) glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 10% volume per volume (v/v) heat inactivated foetal calf serum (FCS; Invitrogen), 5 µg/ml Insulin (Sigma), 10µg/ml Hydrocortisol (Sigma), 20 ng/ml Epidermal Growth Factor (EGF, Sigma), and 20 ng/ml Cholera toxin (Sigma).

Φ amphotropic, Φ ecotropic and Human HEK 293 T/17 and HMF3A cells were grown in DMEM supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% v/v heat inactivated FCS.

#### **2.1.3 Cell culture conditions**

All cell lines were maintained in a 5% CO<sub>2</sub> and 20% oxygen atmosphere. Φ amphotropic, Φ ecotropic, HEK 293 T/17. The 226L 8/13 cell line and 226L 8/13 EcoR Clone 7 cells were maintained at 34°C ±0.5°C, a temperature at which the cells proliferated continuously due to the functional activity of U19tsA58 LT. 226L 8/13 and EcoR Clone 7 temperature shift experiments were performed at 38°C ±0.5°C, a temperature at which U19tsA58 LT was inactivated and the cells became senescent within a period of 7-14 days.

#### **2.1.4 Sub-culturing of cells**

226L 8/13 and EcoR Clone 7 cells were grown until a sub-confluent state was reached (approximately 80% confluence). Medium was then removed and the cells washed

three times with Phosphate Buffered Saline (PBS; Invitrogen) and once with versene-ethylenediaminetetraacetic acid (versene/EDTA; Invitrogen). The cells were detached using trypsin-EDTA (0.25% trypsin and 0.03% EDTA; 1 ml/T75 flask or equivalent; Invitrogen) for 5 mins at 37°C and the trypsin-EDTA was inactivated by adding 9 parts complete medium/1 part trypsin. Cells were then plated at a defined ratio (e.g. 1 in 5 of the total cells), or counted using a haemocytometer and plated at the required density.

HMF3A and HEK 293 cells were sub-cultured as described above, but no versene/EDTA was used and trypsin was only applied for 3 minutes.

For  $\Phi$  amphotropic and  $\Phi$  ecotropic cells no versene/EDTA and trypsin was used but cells were washed off with medium following two PBS rinses.

### **2.1.5 Preservation of frozen cells**

Cells from a sub-confluent T175 flask were trypsinised, resuspended in complete medium and spun down at 485g for 2 mins to remove any traces of trypsin. Cells were resuspended in complete medium supplemented with 10% dimethyl sulphoxide (DMSO; Sigma). 3x 1 ml aliquots were then transferred to cryotubes (Nunc) and frozen at -70°C wrapped in several layers of tissue for insulation. Tubes were transferred into liquid nitrogen after 24 hrs.

### **2.1.6 Recovery of frozen cells**

Cells were removed from liquid nitrogen storage and thawed rapidly at 37°C. 10 ml of complete medium was added to the cells in a 15 ml falcon tube and cells were pelleted at 485g for 2 mins to remove DMSO-containing medium. The cell pellet was resuspended in 10 ml of complete medium, transferred to a T175 cm<sup>2</sup> flask and incubated at the appropriate temperature in a 5% CO<sub>2</sub> and 20% oxygen atmosphere until sub-confluence was reached. Cells were then sub-cultured, as described above.

### **2.1.7 Complementation assay**

226L 8/13 or EcoR Clone 7 were infected with retroviral or lentiviral supernatant and selected with the appropriate antibiotic as described in section 2.2. Following drug selection, when no viable cells remained in non-infected control cultures, drug resistant clones in infected cultures were reseeded at approximately 1,050 cells per cm<sup>2</sup> (which corresponds to 80,000 cells in T75 flasks or 10,000 cells per well in 6-well plates),

shifted to the non-permissive temperature the following day, and incubated at the non-permissive temperature for 2 to 2.5 weeks. Following the incubation period, cells were stained with methylene blue (2% [w/v] methylene blue and 50% ethanol in double-distilled [dd] H<sub>2</sub>O) at room temperature for 10-15 mins.

#### **2.1.8 Crystal violet staining to determine cell growth**

Cells were seeded at 500 cells/well in 96 well plates (Nunc) and grown at the appropriate temperature. Cells were washed twice with PBS and fixed in 200 µl industrial methylated spirit (IMS, VWR) for 10 minutes, after which IMS was taken off and plates were air dried. Plates were then washed once with 200 µl PBS and stained with 100 µl 0.1% crystal violet (10 mg crystal violet from Sigma, 1 ml methanol and 9 ml distilled water) for 20 minutes at room temperature. Plates were washed with tap water until all excess stain was removed and air dried. 100 µl/ well of 33% acetic acid (VWR) were added, plates were placed on an orbital shaker for 10 minutes until stain was evenly distributed throughout the wells, and optical density (OD) was measured at 590 nm on a luminometer.

#### **2.1.9 Senescence associated $\beta$ -galactosidase**

Cultures were fed with full medium the day before the assay which was performed in 6 well plates. Medium was aspirated from the cells, and cells were washed twice with 1 ml of PBS. Then, 1.5 ml of 1 x Fixation Buffer (10 x solution containing 20% formaldehyde, 2% glutaraldehyde, 70.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 14.7 mM KH<sub>2</sub>PO<sub>4</sub>, 1.37 M NaCl, and 26.8 mM KCl) was added and cells were incubated for 6-7 minutes at room temperature. Following this incubation cells were rinsed 3 times with 1 ml of PBS, and 1 ml of the Staining Mixture [1 part Staining Solution, 0.125 parts Reagent B (400 mM Potassium Ferricyanide), 0.125 parts Reagent C (400 mM Potassium Ferrocyanide), 0.25 parts X-gal Solution (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; 40 mg/ml) and 8.5 parts ultrapure water] was added per well. Plates were sealed with parafilm and incubated at 37 °C without CO<sub>2</sub> over night. The following day the Staining Mixture was replaced by PBS and cells were photographed using an Olympus CKX41 microscope.



## **2.2 Retroviral and lentiviral infection protocol**

### **2.2.1 Retroviral and lentiviral constructs**

pLPCX was purchased from BD Biosciences, Retroviral vector pBabepuro-wt LT cDNA and pRetroSuper were provided by O. Gjoerup, University of Pittsburgh, USA; pLPC-12SE1AORI was from S. Lowe, Cold Spring Harbor Laboratory, USA; pBabePuro HPV16 E7 was provided by K. Munger, Harvard Medical School, USA; pLXIPGSEp53 was provided by A. Gudkov, Roswell Park Cancer Institute, USA; pWZLpuro $\epsilon$ -EcoR was from J. Downward, CRUK, UK; pWZL-BlastF was from J. Morgenstern, Millenium Inc., USA; pWZLBlast $\epsilon$ -EcoR was constructed by Dr. Louise Mansfield by subcloning the EcoR gene from pWZLpuro $\epsilon$ -EcoR into pWZL-BlastF; pLPCX-E2F-DB was constructed by Dr. Louise Mansfield by subcloning the E2F-DB gene from pCMV-DB provided by Xin Lu (LICR, UK) into pLPCX; pRetroSuper-p53, pRetroSuper-p21 and pRetroSuper-lamin were constructed by Dr. Louise Mansfield;

Lentiviral shRNAmir silencing constructs from the Open Biosystems human GIPZ lentiviral shRNAmir library, were provided by the UCL shRNA library core facility. Lentiviral Gag/Pol expression vector p8.91 and VSV-G viral envelope expression vector pMDG.2, were provided by G. Towers (UCL, UK) and D. Trono (University of Geneva, Switzerland).

### **2.2.2 Packaging of retroviral constructs**

$\Phi$  amphotropic and  $\Phi$  ecotropic retroviral packaging cells were plated at  $1 \times 10^6$  cells/10 cm<sup>2</sup> plate the day prior to transfection. Cells were transfected the following day (in 10 ml complete medium) with 10  $\mu$ g of retroviral vector DNA and 12  $\mu$ l of FuGENE 6 Transfection reagent (ROCHE). 24 hrs post-transfection, medium was changed using 10 ml fresh medium per plate. 48 and 72 hrs post-transfection, the retroviral supernatant was harvested, filtered through a 0.45  $\mu$ m filter, quickly frozen at -80°C. Frozen aliquots of retroviral supernatant were thawed rapidly at 37°C before use.

### **2.2.3 Retroviral infection**

Cells utilised for lentiviral infection were seeded at  $5 \times 10^5$  cells/T75 cm<sup>2</sup> flask. The following day (at approximately 30% confluence), medium was aspirated, and cells were infected with 5 ml retroviral supernatant in the presence of 8  $\mu$ g/ml polybrene in

15 ml of full medium. Cells were then incubated at 34°C for 24 hrs. The following day, medium was replaced with 15 ml fresh medium and 48 hrs post-infection, antibiotic selection was added (2 µg/ml puromycin or 5 µg/ml blasticidin, where appropriate; Invitrogen), and medium (including antibiotic) was changed every 3-4 days.

#### **2.2.4 Retroviral titration**

Cells were seeded at 64,000 cells per well in 6-well plates and infected with a range of supernatant (0.1 µl, 1 µl, 10 µl, 20 µl and 30µl) following the protocol described above. Selection was discontinued after 5 days and 14 days post-antibiotic selection, medium was removed from the flasks and cells were stained with methylene blue at room temperature for 10-15 mins. Plates were then gently rinsed with water, and cell colonies counted.

#### **2.2.5 Packaging of lentiviral constructs**

HEK 293 T/17 cells were plated at  $6 \times 10^6$  cells/10 cm<sup>2</sup> plate the day prior to transfection. Cells were transfected the following day (in 10 ml of complete medium) with 1.5 µg of the lentiviral vector DNA, 1 µg p8.91 packaging plasmid DNA (gag-pol expresser), 1 µg pMDG.2 packaging plasmid DNA (VSV-G expresser) and 10 µl of Eugene 6 Transfection reagent. 24 hrs post-transfection, medium was changed using 10 ml fresh medium per plate. 48 and 72 hrs post-transfection, the lentiviral supernatant was harvested, filtered through a 0.45 µm filter, and quickly frozen at -80°C. Frozen aliquots of retroviral supernatant were thawed rapidly at 37°C before use.

#### **2.2.6 Lentiviral infection**

Cells utilised for lentiviral infection were seeded at  $2.5 \times 10^5$  cells/T75 flask or 80,000 cells/T25 flask. The following day, medium was aspirated, and cells were infected with lentiviral supernatant in the presence of 8 µg/ml polybrene (Sigma) in full medium. Cells were then incubated at 34°C for 24 hrs. The following day, medium was replaced with fresh medium and 24 hrs post-infection, antibiotic selection was added (6 µg/ml puromycin; Invitrogen), and medium (including antibiotic) was changed every 3-4 days.

#### **2.2.7 Lentiviral titration**

Cells were seeded at 64,000 cells per well in 6-well plates and infected with a range of supernatant (0.1 µl, 1 µl, 10 µl, 20 µl and 30µl) following the protocol described above.

6 µg/ml puromycin was added 24 hours post-infection and selection was discontinued after 5 days. 14 days post-antibiotic selection, medium was removed from the flasks and cells were stained with methylene blue at room temperature for 10-15 mins. Plates were then gently rinsed with water, and cell colonies counted.

## **2.3 Immunocytochemistry**

Cells were plated at on coverslips (VWR #631-0148), grown to the required confluence, fixed and stained and then mounted on slides from VWR (#631-0102). Photos were taken with a Zeiss microscope with an Axio-Vision camera system.

### **2.3.1 $\beta$ -catenin**

The  $\beta$ -catenin antibody was purchased from BD Biosciences (#610154). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then fixed for 15 minutes with freshly prepared 3.5% PFA. They were washed 3 times with PBS and then permeabilised with fresh 0.1% Triton X-100 for 10 minutes. Coverslips were washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:200 in blocking solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.2 $\beta$ -4 integrin**

The  $\beta$  4 integrin antibody was purchased from Chemicon (clone 3E1, #MAB1964). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then moved into a glass dish. Ice-cold acetone was added and aspirated immediately (to avoid crystals formed by freezing PBS), more acetone was added and coverslips were incubated for 2 minutes. They were then washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:100 in blocking

solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.3 E-cadherin**

The e-cadherin antibody was purchased from BD Biosciences (#610182). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then fixed for 15 minutes with freshly prepared 3.5% PFA. They were washed 3 times with PBS and then permeabilised with fresh 0.1% Triton X-100 for 10 minutes. Coverslips were washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:200 in blocking solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.4 Cytokeratin 14**

The cytokeratin 14 antibody was purchased from Abcam (clone LL002, #ab7800). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then fixed with ice-cold methanol is for 5 minutes. Coverslips were washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:100 in blocking solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.5 Cytokeratin 18**

The cytokeratin 18 antibody was purchased from Sigma (clone CY-90, #C8541). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then fixed for 15 minutes with freshly prepared 3.5% PFA. They were washed 3 times with PBS and then permeabilised with fresh 0.1% Triton X-100 for 10 minutes. Coverslips were washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:200 in blocking solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.6 Cytokeratin 19**

The cytokeratin 19 antibody was purchased from Abcam (clone A53-B/A2, #ab7754). When cells were approximately 60% confluent medium was aspirated, they were washed twice with PBS and then fixed with ice-cold methanol is for 5 minutes. Coverslips were washed three times with PBS, blocked with 0.1% BSA in PBS for 15 minutes and washed another three times with PBS before the primary antibody was added at 1:50 in blocking solution (0.1% BSA in PBS) for 1h at room temperature or overnight at 4°C. Coverslips were washed three times with PBS before adding the secondary goat-anti-mouse-antibody (Molecular Probes) at 1:400 with Dapi (Invitrogen; at final concentration 300nM) in blocking solution for 1h at room temperature in the dark. Then, coverslips were washed three times with PBS and mounted using Invitrogen prolong antifade reagent. They were left to dry in the dark and stored at 4°C.

### **2.3.7 BrdU**

Cells were fixed with 4% PFA for 15 mins, washed 3 times with PBS, and permeabilised with 0.1% triton X-100 for 30 mins at room temperature. This was followed by a DNA denaturation step with 2M HCl for 30 mins at room temperature. Cells were washed 3 times with PBS, blocked with 1% BSA in PBS for 15 mins at room temperature, and washed 3 times with PBS. Alexa Fluor 488 conjugated BrdU

(0.2 mg/ml; Clone MoBU-1; Invitrogen) antibody was used at 1:250 in 0.25% BSA for 2h at room temperature. Cells were washed twice with PBS, and then Dapi (1µg/ml) and HSC cell mask at 3µg/ml (H32712; Invitrogen) in PBS were added and plates incubated for 30 minutes at room temperature. This was replaced with fresh PBS and plates were stored at 4°C. Plates were analysed by Cleo Bishop with the InCell 1000 automated microscope (GE Healthcare/Amersham Biosciences, Little Chalfont, UK).

## **2.4 Bacterial manipulation**

### **2.4.1 Bacterial strains**

The JS4 *Escherichia coli* strain (a kind gift from J. Sedivy, Brown University) was used for plasmid manipulation and preparation. JS4 is a *recA1* derivative of MC1061, and has the following genotype: F-araD139,  $\Delta$ (ara, leu)7697,  $\Delta$ (lac) $\chi$ 74, galU, galK, hsdR2 (rk- mk-), mcrA, mcrBC, rpsL (Strr) thi, *recA1*.

### **2.4.2 Media and bacterial growth**

*E. coli* were grown in Luria Base (LB) Broth (25 g/L Luria Broth Base; Invitrogen) or Superbroth (10 g MOPS sodium salt from Sigma, 20 g yeast extract from Oxoid, and 32 g tryptone from Oxoid per litre). 15 g/L of agar (Oxoid) was added when LB agar plates were prepared. To make up the medium, all the components were dissolved in ddH<sub>2</sub>O and autoclaved for 20 mins at 121°C. As appropriate, antibiotics were added: kanamycin (Sigma) was added to a final concentration of 50 µg/ml, ampicillin (Sigma) and carbenicillin (Sigma) were added to a final concentration of 100µg/ml, zeocin (Autogen Bioclear UK Ltd) was added to a final concentration of 25µg/ml, and chloramphenicol was added to a final concentration of 120 µg/ml.

### **2.4.3 Preparation of competent bacteria**

5 ml of LB medium was inoculated with a single bacterial colony and incubated with shaking at 37°C overnight. The overnight culture was then diluted into 500 ml of LB and grown at 37°C with aeration for 2.0-2.5 hrs until the OD reading at 600 nm (OD<sub>600</sub>) reached 1.0-1.2 (mid-exponential phase). Bacteria were harvested by centrifugation at 2000 g for 20 mins, resuspended in 5 ml ice-cold 0.1 molar (M)

calcium chloride ( $\text{CaCl}_2$ ) and incubated on ice for 20 mins. Bacteria were then centrifuged again and resuspended in 5 ml of ice-cold 85:15 solution 0.1 M  $\text{CaCl}_2$  and glycerol. 50  $\mu\text{l}$  aliquots were frozen in pre-chilled 1.5 ml microfuge tubes using liquid nitrogen, and stored at  $-70^\circ\text{C}$ .

#### **2.4.4 Bacterial transformations**

One 50  $\mu\text{l}$  of competent JS4 bacteria can be used for up to 10 transformations. The aliquot of frozen competent JS4 bacteria was thawed on ice. 950  $\mu\text{l}$  ice cold 0.1 M  $\text{CaCl}_2$  was added; 100  $\mu\text{l}$  of this mixture was then added to each DNA to be transformed. The DNA was incubated with the bacteria on ice for 30 minutes followed by heat shock at  $42^\circ\text{C}$  for 90 seconds (sec). Transformations were returned to ice for 2 minutes, after which 1 ml of LB medium was added and the transformations were incubated at  $37^\circ\text{C}$  with shaking for 30 minutes. Cells were concentrated by centrifugation (2000 g for 10 minutes) and resuspended in 100  $\mu\text{l}$  LB medium. Transformations were then plated onto 10  $\text{cm}^2$  pre-warmed LB-agar plates containing 100  $\mu\text{g}/\text{ml}$  final concentration ampicillin or 50  $\mu\text{g}/\text{ml}$  kanamycin and 120  $\mu\text{g}/\text{ml}$  chloramphenicol, or 100  $\mu\text{g}/\text{ml}$  carbenicillin and 25  $\mu\text{g}/\text{ml}$  zeocin where appropriate. Plates were then incubated at  $37^\circ\text{C}$  overnight.

## **2.5 DNA manipulation**

### **2.5.1 Plasmid DNA preparation**

All plasmid preparations (both small scale and large scale preparations) were carried out using QIAGEN kits following the manufacturer's instructions.

#### ***2.5.1.1 Small scale plasmid preparation***

Bacterial stocks were stored at  $-70^\circ\text{C}$  in LB medium containing 15% glycerol. Liquid cultures of bacteria picked from single colonies were grown in a bacterial shaker (vigorous shaking) overnight at  $37^\circ\text{C}$  in 5 ml of LB medium with the appropriate antibiotic. 1.5 ml of culture was then transferred to a 1.5 ml microfuge tube and spun at 17,900 g for 30 sec. The cell pellet was resuspended in 250  $\mu\text{l}$  of solution P1 (50 mM Tris/hydrochloric acid [HCl], pH 8.0, 10 mM EDTA and 100 mg/ml RNase A). 250  $\mu\text{l}$

of solution P2 (200 mM sodium hydroxide [NaOH] and 1% sodium dodecyl sulphate [SDS]) was added and gently mixed by inverting the 1.5 ml microfuge tube 4-6 times. To the same 1.5 ml microfuge tube, 350 µl of solution N3 (3.0 M sodium acetate, pH 5.5) was added and immediately mixed by inverting the 1.5 ml microfuge tube 4-6 times. The mixture was then spun in a microfuge for 10 minutes at 17,900 g and the supernatant transferred to a QIAprep column. The column was centrifuged for 30 sec at 17,900 g then the flow-through was discarded. The column was then washed with 0.5 ml of PB buffer (QIAprep Spin Miniprep kit, QIAGEN) and then 0.75 ml of PE buffer (QIAprep Spin Miniprep kit, QIAGEN). DNA was then eluted with 50 µl of EB elution buffer (QIAprep Spin Miniprep kit, QIAGEN). All solutions used were from the QIAfilter Plasmid Mini kit, QIAGEN.

#### ***2.5.1.2 Large scale plasmid preparation***

200 ml of superbrot medium containing the appropriate antibiotic was inoculated with an overnight culture of bacteria and grown overnight at 37°C with vigorous shaking. Bacteria were harvested at 6000 g for 15 minutes at 4°C using an SLA 1500 rotor and Sorvall RC5C centrifuge. The cell pellet was resuspended in 10 ml of resuspension buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 100 µg/ml RNase A, stored at 4°C). 10 ml of lysis buffer P2 (200 mM NaOH and 1% SDS) was added and, after a 5 minute incubation step at room temperature, 10 ml of ice-cold neutralisation buffer P3 (3mM potassium acetate pH 5.5) was added and the mixture was directly applied to a QIAfilter Cartridge. The QIAfilter Cartridge was incubated at room temperature for 10 minutes before the cell lysate was filtered and directly applied to a previously equilibrated QIAGEN-tip 500 column (equilibration buffer QBT: 750 mM NaCl, 50 mM MOPS [3 – [N-morpholino] propanesulphonic acid] pH 7.0, 15% ethanol [v/v] and 0.15% Triton X-100) and allowed to enter the resin by gravity. The column was washed twice with 30 ml of wash buffer QC (1 M NaCl, 50 mM MOPS pH 7.0 and 15% ethanol). DNA was then eluted with 15 ml of elution buffer QF (1.25 M sodium chloride [NaCl], 50 mM Tris-HCl pH 8.5 and 15% ethanol) and precipitated in 10.5 ml of isopropanol at room temperature. Centrifugation was performed at 15,000 g for 30 minutes at 4°C using the SS34 rotor and Sorvall RC5C centrifuge. The DNA pellet was washed with 70% ethanol then centrifuged again at 15,000 g for 10 minutes. The supernatant was removed and the DNA pellet was air dried for 5 minutes. DNA was



resuspended in 100-200µl Tris-HCl-EDTA (TE), depending on the size of the pellet, in a 1.5 ml microfuge tube. All solutions used were from the QIAfilter Plasmid Maxi kit, QIAGEN.

### **2.5.2 DNA quantification**

To determine DNA concentration, the OD of the solution was measured at 260 nm (OD<sub>260</sub>) using a Nanodrop 1000 from Thermo scientific. DNA concentration was calculated using the relationship: 1 OD unit at 260 nm = 50 µg/ml DNA

### **2.5.3 Agarose gel electrophoresis**

DNA was loaded with 1x DNA loading buffer (2.5% Ficoll, 0.04% [w/v] bromophenol blue and 0.04% Xylene) and fractionated by electrophoresis on 1-2% (w/v) agarose (Invitrogen) gels as appropriate, prepared in 1x TAE (40 mM Tris-acetate and 2 mM EDTA) with 1 µg/ml ethidium bromide (BDH). Electrophoresis in 1x TAE was carried out in electrophoresis tanks and DNA fragments were separated at a constant voltage of 100 Volts (V) for a minimum of 20 minutes. Samples were loaded alongside 5 µl 1kb+ DNA ladder (Invitrogen). Ethidium bromide stained DNA fragments were then visualised on a UVP (Dual intensity UV trans-illuminator), and an image was saved.

### **2.5.4 Recovery of shRNA inserts from cells expressing pSM2 or pGIPZ constructs**

#### ***2.5.4.1 Genomic DNA extraction***

Genomic DNA was extracted from a near confluent well of 6 well plates using the QIAamp DNA Blood Mini kit (Qiagen). Medium was removed and the monolayer of cells was washed three times with PBS and once with versene/EDTA. The monolayer was detached using trypsin-EDTA (0.25% [v/v] trypsin and 0.03% [weight per volume [w/v]] EDTA; 0.5 ml/well) for 10 minutes at 37°C and the trypsin-EDTA was inactivated by adding 10 ml of PBS. Cells were spun down at 485 g, resuspended in 1ml PBS, transferred to a 1.5 ml microfuge tube, spun down at 485 g again to remove any residual trypsin, and resuspended in 200 µl PBS. 20 µl of QIAGEN Protease (QIAamp DNA Blood Mini Kit, Qiagen) was added, followed by 200 µl Buffer AL (QIAamp DNA Blood Mini Kit, Qiagen). The contents were mixed by pulse-vortexing for 15 sec, followed by incubation at 56°C for 10 minutes. The 1.5 ml microfuge tube was

centrifuged briefly then 200 µl ethanol (96-100%) was added, followed by pulse-vortexing for 15 sec. The mixture was then applied to a QIAamp Spin Column (QIAamp DNA Blood Mini Kit, Qiagen) and centrifuged at 10,000 g for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube then washed with 500 µl Buffer AW1 (QIAamp DNA Blood Mini Kit, Qiagen) and centrifuged at 10,000 g for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube then washed with 500 µl Buffer AW2 (QIAamp DNA Blood Mini Kit, Qiagen) and centrifuged at 10,000 g for 3 mins. The QIAamp Spin Column was then placed in a clean 1.5 ml microfuge tube and 200 µl Buffer AE (QIAamp DNA Blood Mini Kit, Qiagen) was added. Following incubation at room temperature for 1 min, the 1.5 ml microfuge tube was centrifuged at 10,000 g for 1 min.

#### ***2.5.4.2 PCR amplification of pSM2 sequences from genomic DNA***

200 ng genomic DNA was used in a 50 µl PCR reaction that contained 1 µl each of primers pSM2longForward and pSM2longReverse (both at 6.6 µM), 5 µl 10x KOD buffer (Novagen), 5 µl KOD dNTPs (Novagen), 3µl KOD MgSO<sub>4</sub> (Novagen), and 1 µl KOD Hot Start DNA Polymerase (Novagen). An initial denaturation step at 95°C for 2 mins was performed before PCR amplification. PCR amplification parameters were denaturation at 95°C for 30 sec; annealing at 59°C for 30 sec; extension at 70°C for 30 sec, and a final extension of 10 minutes at 70°C after the last cycle. 40 cycles were used in total. 5 µl of each PCR reaction was then resolved alongside 5 µl 1kb+ DNA ladder (Invitrogen) on a 1.4% agarose gel to check for the generation of the 438 bp PCR products that could be visualised on a UVP.

#### ***2.5.4.3 PCR amplification of pGIPZ sequences from genomic DNA***

250-300 ng genomic DNA was used in a 50 µl PCR reaction that contained 3 µl each of a forward primer and a reverse primer provided by Open Biosystems (both at 5pmol/µl), 5 µl 5M Betaine (Sigma Aldrich), 5 µl 10x KOD buffer (Novagen), 5 µl KOD dNTPs (Novagen), 2µl KOD MgSO<sub>4</sub> (Novagen), and 1µl KOD Hot Start DNA Polymerase (Novagen). An initial denaturation step at 94°C for 2 mins was performed before PCR amplification. PCR amplification parameters were denaturation at 94°C for 15 seconds; annealing at 55°C for 30 seconds; and extension at 68°C for 1 minute. 40 cycles were used in total. 5 µl of each PCR reaction was then resolved alongside 5 µl 1kb+ DNA

ladder (Invitrogen) on a 1.4% agarose gel to check for the generation of 561 base pair PCR products that could be visualised on a UVP.

#### ***2.5.4.4 Preparation of PCR products for sequencing***

The PCR product was cleaned up using the micro-CLEAN system (Microzone Ltd). PCR products were loaded on a 96 well plate and equal volumes of microCLEAN were added to the samples (50 µl). Samples were mixed and incubated at room temperature for 5 minutes. Plates were spun at 3000 g for 40 minutes. They were then placed upside down onto tissue paper in the centrifuge holder and pulse centrifuged up to 40 g for 30 seconds. Pellets were resuspended in appropriate volume of ddH<sub>2</sub>O (30-100 µl) as determined by intensity of the band on the agarose gel (see 2.5.4.3).

#### ***2.5.4.5 DNA sequencing***

DNA sequencing was carried out by the genetics group within the MRC Prion unit.

## **2.6 RNA manipulation**

### **2.6.1 RNA isolation**

Cultures grown in T75 cm<sup>2</sup> flasks (or T180 cm<sup>2</sup> flasks if preparing RNA for microarray analysis) were fed with fresh medium the day prior to RNA extraction and were harvested at no greater than 80% confluence on the day of RNA extraction. Medium was removed and 600µl of Buffer RLT (RNeasy Mini Kit, Qiagen; containing 1% β-mercaptoethanol) were added to cultures to lyse the cells. The lysate was applied to a QIAshredder spin column and spun for 2 minutes at maximum speed to homogenise the sample which increases RNA yield. Then, 600µl 70% ethanol was added to the lysate, which was then mixed by pipetting. The lysate was then applied to an RNeasy Mini spin column and centrifuged for 15 seconds at 8000 g and the flow through was discarded. 350 µl Buffer RW1 (RNeasy Mini Kit, Qiagen) were added to the RNeasy spin column and it was spun at 8000 g for 15 seconds. To remove possible DNA contamination, 10 µl DNase I stock solution (RNeasy Mini Kit, Qiagen ) was added to 70 µl Buffer RDD (RNeasy Mini Kit, Qiagen), and 80 µl of the mix was applied to the RNeasy spin column membrane, and incubated for 15 minutes at room temperature. Then, 350 µl

Buffer RW1 were added to the RNeasy spin column and it was centrifuged for 15 s at 8000 g. This was followed by 2 washes with 500 µl Buffer RPE (RNeasy Mini Kit; diluted 1 in 4 parts in 100% ethanol), the first wash spun for 15 seconds at 8000 g, and the second wash for 2 minutes at 8000 g. The RNA was eluted with 50 µl RNase-free water for 1 minute at 8000 g.

### **2.6.2 RNA quantification**

To determine RNA concentration, the OD of the solution was measured at 260 nm (OD<sub>260</sub>) using a Nanodrop 1000 from Thermo Scientific. RNA concentration was calculated using the relationship: 1 OD unit at 260 nm = 40 µg/ml RNA

### **2.6.3 Reverse transcription (RT) of RNA**

cDNA was reverse transcribed from equal amounts (2 µg) of total RNA using MoMuLV reverse transcriptase. The SuperScript II RT kit (Invitrogen) was used, according to manufacturer's instructions. RNA was added to 0.5 µg of oligo dT primer (Promega) and 0.5 mM dNTPs (Promega) then denatured at 65°C for 5 mins followed by quick chilling on ice. 1 µl ribonuclease inhibitor (Promega) and 1 µl 0.1M DTT were then added to the mixture, together with 2 µl of 5x First-Strand Buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl and 15 mM MgCl<sub>2</sub>), and incubated at 42°C for 2 mins. 200 units of MoMuLV reverse transcriptase enzyme was added and the reaction was incubated at 42°C for 50 mins before heat inactivation at 70°C for 15 mins to denature the RNA-DNA duplex and inactivate the reverse transcriptase. The resultant cDNA was stored at -20°C.

### **2.6.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

All PCR reactions were carried out in a total volume of 25 µl and contained 10 µl of a 1:10 dilution of the cDNA generated by RT reaction, 1 µl of each oligonucleotide primer (10 µM), 2.5 units Taq DNA polymerase (Promega), 2.5 µl 10x PCR buffer (10 mM Tris HCl pH 9, 50 mM KCl and 0.1% [w/v] Triton X-100; Promega), 1 µl 10 mM dNTPs (Promega) and 2 µl 25 mM MgCl<sub>2</sub> (Promega). The PCR reaction was performed in a PTC-200 Peltier Thermal cycler thermocycler. A 1 minute, 94°C denaturation step was used before amplification. Cycling conditions were denaturation at 94°C for 1 min, annealing at the specific temperature for each primer pair for 1 min,

extension at 72°C for 1 min, and a final extension of 5 mins at 72°C at the end of the last cycle; 35 cycles were performed. For each PCR reaction, a control RT reaction containing no reverse transcriptase (RT- control) was included to check for DNA contamination of the RNA samples or reaction mixture. PCR products were resolved alongside 5 µl 1kb+ DNA ladder (Invitrogen) on 2% agarose gels, depending upon the size of the expected PCR product. Ethidium bromide stained DNA fragments were visualised on a UVP and images were saved.

Annealing temperature for the primer sets:

LOC219321 (53095): 56°C; LOC343425 (25465): 56°C; LOC351347 (127153): 57°C; FLJ31301 (68969): 56°C; LOC346321 (145373): 58°C; LOC349975 (109096): 56°C;

### **2.6.5 Real-Time quantitative RT-PCR**

The TaqMan Gene Expression Cells-to-CT Kit from Applied Biosystems (AB) was used to perform quantitative RT-PCR. The following TaqMan Gene Expression Assays (AB) were used: p21: Hs00355782\_m1; CCNL1: Hs00220399\_m1; FOXA1: Hs00270129\_m1; Rab23: Hs00212407\_m1; SPOPL: Hs01100158\_m1

### ***RNA isolation***

The day before lysis 10,000 cells/well were seeded in 96 well plates (Nunc). The following day cells were washed with cold PBS, and 50 µl of Lysis Solution (AB) was added per well and mixed. Following 5 minutes of incubation at room temperature, 5 µl of Stop Solution (AB) was added, mixed and incubated for 2 minutes at room temperature. Lysates were stored at -20°C until Reverse Transcription was performed.

### ***Reverse Transcription***

10 µl of the lysate was used for the RT reaction and was mixed with 40 µl of master mix. For each reaction the master mix was prepared by mixing 25 µl of 2x RT Buffer (AB), 2.5 µl of 20x RT Enzyme (AB) and 12.5 µl of nuclease free water. The reactions were incubated at 37°C for 60 minutes followed by heat inactivation of the RT enzyme at 95°C for 5 minutes. The resultant cDNA was stored at -20°C.

## ***Real-Time PCR***

cDNA amplification was carried out in a 20 µl reaction containing 4 µl of cDNA, 10 µl 2x TaqMan Gene Expression Master Mix (containing AmpliTaq Gold DNA Polymerase, AmpErase UDG [Uracil-DNA Glycosylase], dNTPs with dUTP, ROX passive reference dye, and optimised buffer components; AB), 1 µl 20x TaqMan Gene Expression Assay (containing primer sets and probes for the gene of interest; AB), 1 µl 20x ACTB TaqMan Gene Expression Assay (containing primer sets and probes for the endogenous control gene  $\beta$ -Actin; AB) and 4 µl nuclease-free water. Reactions were performed in a MicroAmp Optical 96-Well Reaction Plate (AB) covered with a MicroAmp Optical Cap (AB). Real-time PCR quantitation was measured using the Applied Biosystems '7500 Fast Real-Time PCR System' according to the manufacturer's instructions. Before amplification, a 2 mins incubation step at 50°C was used to optimise UDG activity, followed by a 10 mins enzyme activation step at 95°C. Amplification parameters were: denaturation at 95°C for 15 sec, followed by a combined annealing and extension step at 60°C for 1 min. 40 cycles were used in total.

## **2.7 Protein analysis**

### **2.7.1 Preparation of total protein extracts**

Cultures grown in T75 flasks were fed with fresh medium the day prior to lysis and were harvested at no greater than 80% confluence on the day of lysis. For lysis, cells were washed twice with cold 1x PBS, and 0.5 ml of 1x radioimmunoprecipitation (RIPA) lysis buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) was added to each T75 flask. 2 µl of Protease Inhibitor Cocktail (2 mM 4-[2-aminoethyl] benzenesulphonyl fluoride [AEBSF], 1 mM EDTA, 130 µM Bestatin, 14 µM E-64, 1 µM Leupeptin and 0.3 µM Aprotinin; Sigma) was added per 1 ml of lysis buffer used. Cells were incubated on ice for 30 mins then scraped and transferred to a 1.5 ml microfuge tube. Lysates were passed three times through a 21-gauge needle to shear the DNA then centrifuged at 8000 g for 30 mins at 4°C. The supernatant from each lysis reaction was transferred to a fresh 1.5 ml microfuge tube, aliquoted and stored at -80°C.

### **2.7.2 Determination of protein concentration**

The Bio-Rad Protein Assay was used to determine protein concentration. The Dye Reagent (Bio-Rad) was prepared by diluting 1 part Dye Reagent concentrate with 4 parts Distilled De-Ionized water (DDI water). BSA standards were prepared in PBS (0 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, and 0.5 mg/ml) and 10 µl of the protein samples to be analysed were diluted 1 in 10 in PBS. Samples were diluted as the linear range of this assay is from 0.05 mg/ml to approximately 0.5 mg/ml; therefore undiluted sample concentrations would be too high for the assay. 10 µl of each sample and standard were used per reaction, and 200 µl of the diluted Dye Reagent were added in 96 well plates (Nunc). Reactions were mixed and incubated at room temperature for 10 minutes, and then absorbance was measured at 595 nm on a luminometer. A BSA standard curve was established, the regression coefficient was calculated and the unknown sample concentrations determined.

### **2.7.3 Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis**

16% and 10% gels (Invitrogen) were used as stated. 35 µg of each cell lysate (unless otherwise stated) was heated at 90°C for 5 mins with 2x Laemmli sample buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.008% bromophenol blue and 0.260 mM Tris-HCl, pH 6.8) and fractionated by SDS-PAGE. Electrophoresis was carried out at a constant voltage of 100-150 V in running buffer (25 mM Tris, 190 mM Glycine, 0.1% [w/v] SDS). Proteins were fractionated alongside broad-range pre-stained Kaleidoscope protein size marker (Bio-Rad Laboratories).

### **2.7.4 Western blotting of SDS-PAGE**

Following separation via SDS-PAGE, proteins were transferred to a nitrocellulose membrane, Hybond-c extra (Amersham Life Science) by electrophoretic transfer in a wet tank blotting system (Bio-Rad Laboratories Trans-Blot cell). The transfer was carried out in transfer buffer (25 mM Tris, 190 mM glycine and 20% [v/v] methanol) for 3 hrs at a constant voltage of 25 V at room temperature. The nitrocellulose membrane was then blocked by incubation in 5% (w/v) skimmed milk powder (Marvel, Premier Brands) and 0.005% (v/v) Tween-20 (BDH Laboratory) in PBS at room

temperature for 1 hr. The filter was then incubated for 1 hr at room temperature, or overnight at 4°C with the primary antibody diluted in 5% milk in PBS with 0.05% Tween or 3% BSA in PBS with 0.05% Tween at the indicated dilutions (as described below). The filter was then washed three times (15 mins each at room temperature) in PBS with 0.05% Tween prior to incubation with horseradish peroxidase (HRP) conjugated secondary antibody (GE Healthcare UK limited) diluted 1:10,000 in 5% milk in PBS with 0.05% Tween for 1 hr. Following three further washes (15 mins each at room temperature) in PBS with 0.05% Tween, the filters were developed in HRP detection reagents for 60 sec, according to manufacturer's instructions (Amersham ECL Western blotting detection reagents, Amersham Pharmacia Biotech). The membrane was then wrapped with Saran-wrap and exposed to an auto-radiographic film for times varying from 10 sec to 2 hrs (Amersham Hyperfilm ECL). Films were developed with an AGFA X-ray film processor.

#### **2.7.5 Antibodies used**

anti- $\beta$ -tubulin mouse monoclonal antibody (clone 2-28-33) was purchased from Sigma; anti-p21 mouse monoclonal antibody (clone SX118) was purchased from BD Biosciences; CCNL1 antibody raised in rabbit was a kind gift from Dr. Lahti; FOXA1 antibody (ab23738) was purchased from Abcam; Rab23 antibody raised in rabbit was a kind gift from Professor Bor Luen Tang.

The antibodies were diluted and incubated for Western blot analysis as follows:

$\beta$ -tubulin (1:2000 in 5% milk in PBS with 0.05% Tween for 1 hour at room temperature); p21 (1 in 250 in 5% milk in PBS with 0.05% Tween at 4°C over night); CCNL1 (1 in 1000 in 5% milk in PBS with 0.05% Tween for 1 hour at room temperature); FOXA1 (1 in 500 in 5% milk in PBS with 0.05% Tween at 4°C over night); Rab23 (1 in 500 in 3% BSA in PBS with 0.05% Tween at 4°C over night);



## **2.8 shRNA library screens**

### **2.8.1 pSM2 viral production, titrations and confidence intervals**

10µg of each pool were packaged using the Φ ecotropic retroviral packaging system. Of each harvest, 1ml of viral supernatant was aliquoted separately to use for titrations. Titrations were performed on the EcoR Clone 7 cells (as described in 2.2.4) to determine the amount of virus required for a MOI of 0.1, to achieve single inserts per cell. To determine the number of infectious events required to achieve a full saturation screen, the following calculation was used:

$$\ln (1-0.99) / \ln (1-1/(\text{Library Size}))$$

(Nolan laboratory; <http://www.stanford.edu/group/nolan/screens/screens.html>)

This indicated that for pools consisting of approximately 200 constructs, 919 infectious events were required to be 99% confident that every construct is assayed.

### **2.8.2 pGIPZ titrations and confidence intervals**

The library consisted of 7 pools, each containing approximately 9,000 constructs. Using the formula  $\ln (1-0.99) / \ln (1-1/(\text{Library Size}))$ , it was determined that 41,444 infectious events were required per pool.

#### ***2.8.2.1 Determination of the titer of the pGIPZ pools in EcoR Clone7 cells***

For each pool the viral titer in HEK 293 cells was known. To determine the titer of each pool in our target cells, a non-silencing control whose titer in 293 cells was known was titrated in EcoR Clone 7 cells:

The day before transduction, a 24 well tissue culture plate with EcoR Clone 7 cells was seeded at 13,000 cells per well in their respective media. Dilutions of the Non-silencing control shRNAmir viral stock were made in a round bottom 96 well plate using serum free E93 medium. 4 replicates of dilutions of the viral stock were made, each a series of 5-fold dilutions to reach a final dilution of 390625-fold. To each well (rows A, B, C, and D 1-8) 80 µl of serum free medium were added, and 20 µl of thawed Non-silencing control shRNAmir virus stock to the first well in each row (5 fold dilution). Contents were mixed with a pipette, and with new pipette tips, 20 µl from each well of column 1

were transferred to the corresponding well in column 2. Again, contents were mixed and with new pipette tips, 20  $\mu$ l from each well of column 2 were transferred to the corresponding well in column 3. This was repeated from columns 3 through 8, pipetting up and down 10-15 times and changing pipette tips between each dilution. Culture medium was removed from the cells in the 24 well plate and 225  $\mu$ l of serum free medium were added to each well. Then, cells were transduced by adding 25  $\mu$ l of diluted Non-silencing control shRNAmir virus from the 96 well plate to a well on the 24 well destination plate containing the cells. After transduced cultures were incubated at 34°C for 4 hours, 1ml of E93 medium (normal serum concentration) was added. Puromycin selection with 6  $\mu$ g/ml was started 24 hours post-transduction. Cells were grown for 14 days, stained with methylene blue, and colonies were counted to determine the titer of the non-silencing control in the target cells. This was then used to determine the titer of the 7 pools in the EcoR Clone 7 cells.

Non-silencing control titered by Open Biosystems in HEK 293

$\div$  Non-silencing control shRNAmir titered by user in their respective cell line and medium

= Relative transduction efficiency

Titer for pools titer in HEK 293 cells by Open Biosystems

$\div$  Relative transduction efficiency

= Titer of pools in EcoR Clone 7 cells

### **3 Results - Characterisation of the 226L 8/13 cells**

The 226L 8/13 cells were developed by Parmjit Jat and Mike O'Hare to study cellular senescence, a programme of irreversible cell cycle arrest which cells must overcome to become cancerous, which has been demonstrated *in vivo* (Chen et al., 2005; Cosme-Blanco et al., 2007; Feldser and Greider, 2007; Hanahan and Weinberg, 2011). 226L 8/13 cells were derived from mammary luminal epithelial cells, and they were conditionally immortalized, so that they can be made to arrest in a synchronised manner. This synchronised arrest facilitates the study of senescence. It is of importance as one of the main stumbling blocks in studying the finite proliferative life span has been the absence of suitable model systems because of the asynchrony as well as the complexity of this process in heterogeneous cell populations that are typically used to study this process by serial subcultivation *in vitro*.

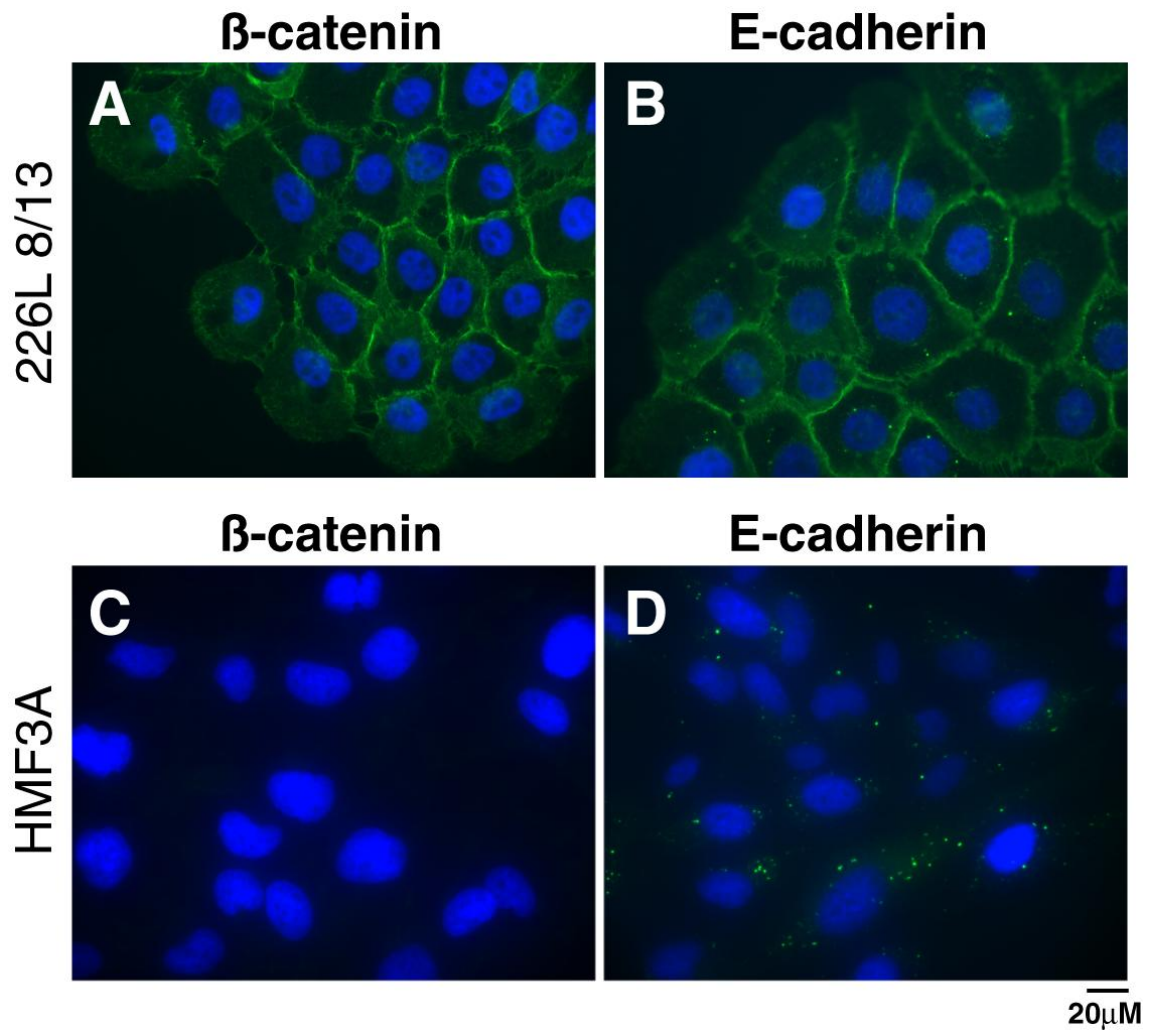
#### **3.1 226L 8/13 cells express the epithelial markers $\beta$ -catenin and E-cadherin**

Cellular context is highly significant in the development of cancer, and most cancers originate in epithelial cells. Cancers of epithelial cells are called carcinoma and make up approximately 85% of all cancers. Other types of cancers are sarcomas (malignant tumours derived from connective tissue, or mesenchymal cells), leukaemias and lymphomas (derived from haematopoietic cells), germ cell tumours, blastomas (tumours which resemble and immature or embryonic tissue), and tumours derived from other body tissues (the biggest group of these are melanoma). Considering the importance of cellular context and that most cancers originate in epithelial cells, the conditionally immortalized 226L 8/13 cells were developed from mammary luminal epithelial cells, to study cellular senescence in an epithelial cell model. Most studies investigating cellular senescence are done in fibroblasts, and it is of relevance to find out if the pathways that regulate senescence in fibroblasts are the same in epithelial cells.

To confirm that 226L 8/13 cells had not lost their epithelial phenotype due to the immortalization process or culturing conditions, they were stained for two epithelial markers, namely  $\beta$ -catenin and E-cadherin. 226L 8/13 cells were seeded at 10,000 cells

on coverslips in 24-well plates. When cells reached about 60% confluence they were fixed and stained for  $\beta$ -catenin and E-cadherin. Dapi was used to stain nuclei. Cells stained positive for both markers (Figure 3.1, A and B). HMF3A mammary fibroblasts were used as a negative control, because fibroblasts do not express these markers. HMF3A cell were negative for both markers (Figure 3.1, C and D).  $\beta$ -catenin was observed mainly on the membrane at cell-to-cell boundaries, which correlates with the fact that it plays a crucial role in cell–cell adhesion. Also, it is in accordance with the finding that normal epithelia display cell boundary staining for beta-catenin (Hao et al., 1997). Some cytoplasmic staining was also observed. Cytoplasmic localization of beta-catenin has been associated with poor outcome in breast cancer patients, but some cytoplasmic staining is normal (Lopez-Knowles et al., 2010). E-cadherin, a transmembrane glycoprotein, is strongly expressed in the cytoplasmic membrane of normal ductal epithelial cells in the mammary gland (Bukholm et al., 2000), and in 226L 8/13 cells E-cadherin staining was observed in the plasma membrane as expected. Some punctate staining was observed with the E-cadherin antibody, which was also observed in the negative control cells. This punctate staining was not observed in the control using the secondary antibody alone (data not shown). Furthermore the same secondary antibody was used for  $\beta$ -catenin staining where the punctate staining was not observed; Therefore it was most likely not an artefact caused by the secondary antibody. The punctate staining occurred in all repeat experiments.

These results show that 226L 8/13 cells have retained their normal epithelial phenotype and therefore represent a good model system to study epithelial cells.



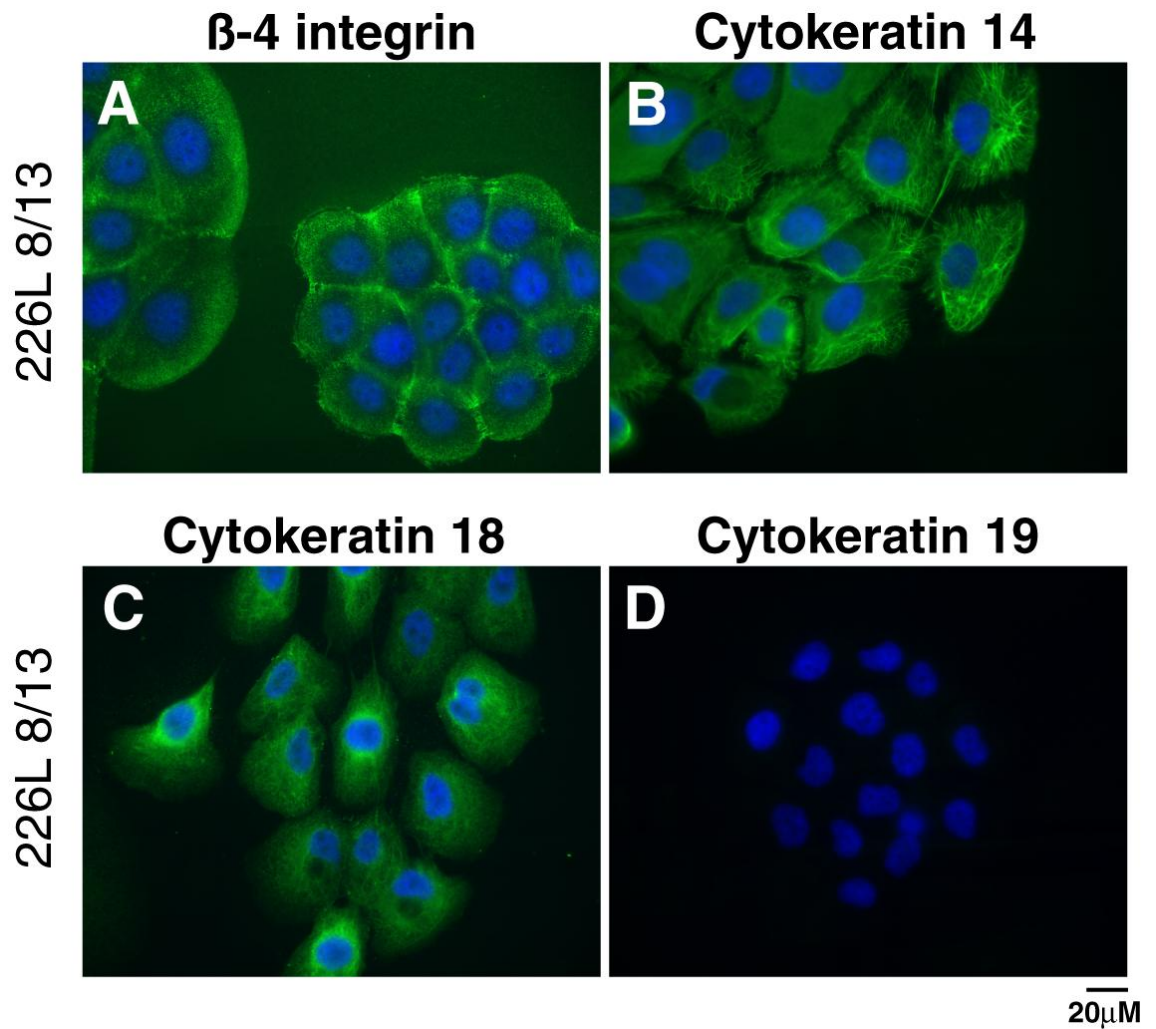
**Figure 3.1: 226L 8/13 cells express the epithelial markers  $\beta$ -catenin and E-cadherin**

226L 8/13 cells were stained for the epithelial markers  $\beta$ -catenin and E-cadherin. (A) 226L 8/13 cells express  $\beta$ -catenin. (B) 226L 8/13 cells express E-cadherin. Negative control HMF3A cells were negative for (C)  $\beta$ -catenin and (D) E-cadherin

### 3.2 226L 8/13 cells express luminal and basal markers

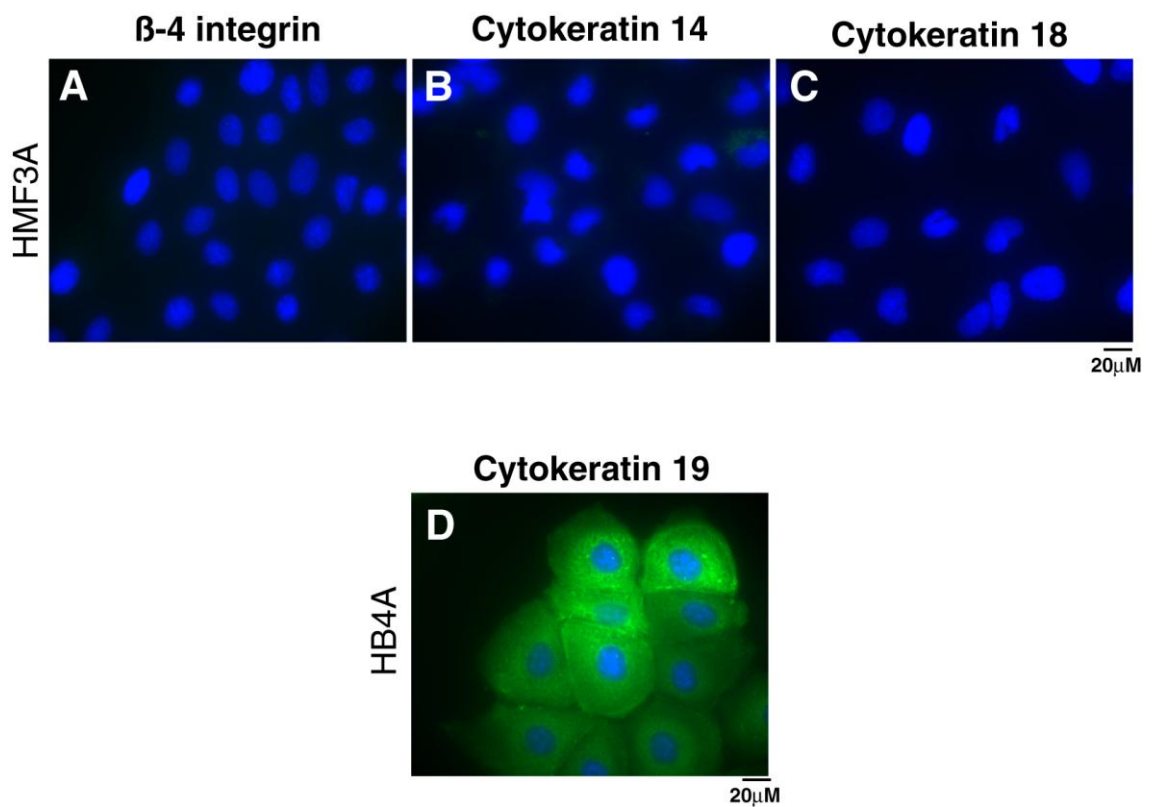
Two types of epithelial cells are found in the human breast, myoepithelial and luminal-epithelial cells. The most common subtypes of breast cancer are luminal subtypes, but cancers that express myoepithelial (basal) markers have a poorer prognosis (El Rehim et al., 2004). The luminal epithelial and myoepithelial cells in the human mammary gland ductal system can be distinguished by the pattern of keratin expression (Taylor-Papadimitriou et al., 1989). Cytokeratins, or keratins, are intermediate filament-forming proteins that provide mechanical support and fulfil a variety of additional functions in epithelial cells. 226L 8/13 cells were stained for the luminal markers cytokeratin 18 and cytokeratin 19, and the basal marker cytokeratin 14. When 226L 8/13 cells were initially isolated, they were negatively selected against the basal marker  $\beta$ -4 integrin, a plasma membrane protein with a long cytoplasmic domain; therefore the cells were also stained for  $\beta$ -4 integrin. Cells stained positive for both basal markers (Figure 3.2a, A and B) and the luminal marker cytokeratin 18 (Figure 3.2a, C). However, they were negative for the luminal marker cytokeratin 19 (Figure 3.2a, D). The intermediate filaments were visible in cytokeratin 14 and 18 staining and both had a typical appearance (O'Hare et al., 1991), and the  $\beta$ -4 integrin staining also had a typical appearance. HMF3A mammary fibroblasts were used as negative controls, and they were negative for all 4 markers (Figure 3.2b, A, B and C). HB4A cells, which are immortalized derivatives of luminal cells and have been shown to express cytokeratin 19 (O'Hare et al., 1991), were used as a positive control for cytokeratin 19 staining; HB4A cells stained positive for cytokeratin 19, confirming that the staining protocol and antibody worked. (Figure 3.2b, D).

In conclusion, even though immunomagnetically sorted luminal cells were used to generate the 226L 8/13 cells, cells had a mixed expression profile, expressing markers of both luminal and basal cells, and not expressing the luminal marker cytokeratin 19.



**Figure 3.2a: 226L 8/13 cells express luminal and basal markers**

226L 8/13 cells were stained for the basal markers  $\beta$ -4 integrin and cytokeratin 14, and for the luminal markers cytokeratin 18 and cytokeratin 19. They express (A)  $\beta$ -4 integrin, (B) cytokeratin 14 and (C) cytokeratin 18. (D) 226L 8/13 cells do not express cytokeratin 19.



**Figure 3.2b: Controls for  $\beta$ -4 integrin, cytokeratin 14, cytokeratin 18 and cytokeratin 19 staining**  
 Negative control HMF3A cells were negative for (A)  $\beta$ -4 integrin, (B) cytokeratin 14, and (C) cytokeratin 18. (D) Positive control HB4A cells were positive for cytokeratin 19

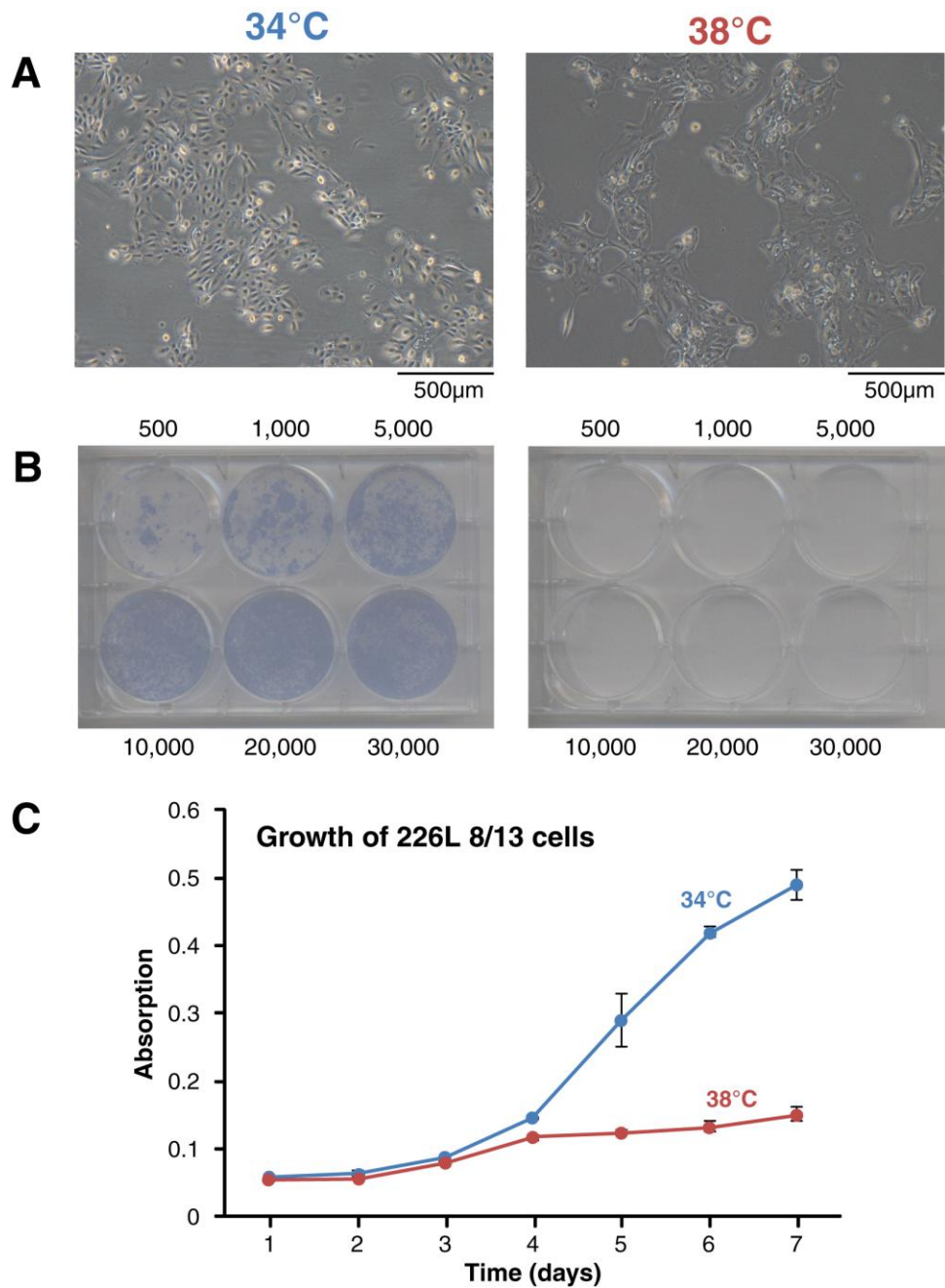


### **3.3 226L 8/13 cells grow at the permissive temperature, 34°C, but they arrest when incubated at the non-permissive temperature, 38°C**

To determine the ability of the 226L 8/13 cells to proliferate at 34°C and 38°C, cells were seeded and incubated at 34°C and 38°C for 7 days. Cultures grown at the permissive temperature had to be split once, whereas cultures at the non-permissive temperature ceased to grow and did not need to be split. After 7 days at each temperature cultures were photographed. Representative images are shown in Figure 3.3 A. Cultures grown at the permissive temperature show the normal morphology of dividing cells, with a few cells showing senescent morphology in these cultures. They grew in islands, which is typical of epithelial cells, and also have the characteristic cobblestone morphology of epithelial cells. 226L 8/13 cells incubated at the non-permissive temperature acquired a phenotype typical of senescent cells; they were enlarged with a flattened morphology and vacuole rich cytoplasm.

To further confirm that the cells undergo growth arrest upon inactivation of the U19TSA58 LT antigen, cells were seeded at 500, 1000, 5000, 10000, 20000, and 30000 cells in 6 well plates and grown for 2 weeks at 34°C and 38°C and then stained with methylene blue; shown in Figure 3.3 B. Blue stained cells were clearly visible at every concentration on the plate incubated at 34°C. With 20,000 and 30,000 seeded cells a near confluent layer of cells had formed, the 10,000 seeded cells had grown to approximately 90% confluence and the 5,000 cells to approximately 50% confluence. The 1,000 cells had grown to about 30% confluence, and the 500 cells to approximately 10 %. No blue colonies were visible on the plate incubated at 38°C.

Further growth assays were carried out by seeding 500 cells per well in 96 well plates, incubating them at 34°C and at 38°C, and staining 3 wells for each temperature every day from day 1 (24 hours after cells were seeded) to day 7. Plates were fixed, stained with crystal violet, and optical density was measured at 590 nm in a luminometer. At 38°C cells grew at approximately the same rate as at 34°C until day 3, when they started slowing down and growth ceased (Figure 3.3 C).



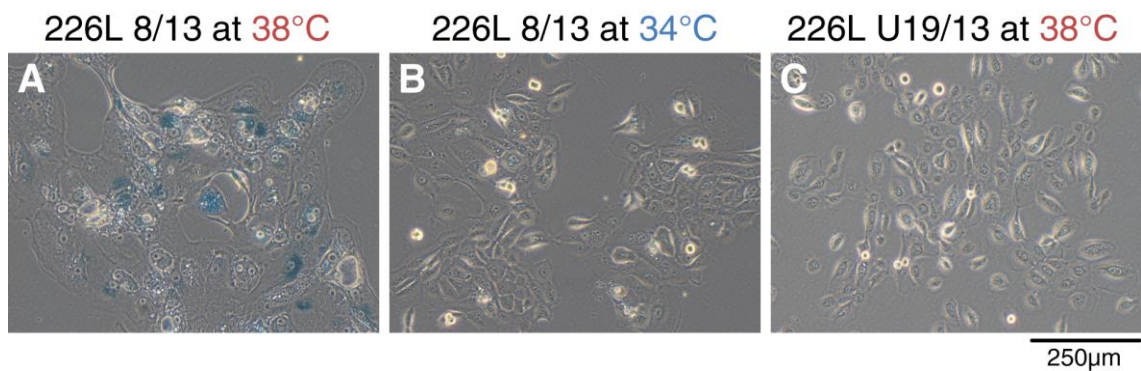
**Figure 3.3: Growth of 226L 8/13 cells at 34°C and 38°C**

(A) 226L 8/13 cells incubated at the permissive temperature showed the normal morphology of dividing epithelial cells. 226L 8/13 cells incubated at the non-permissive temperature acquired a phenotype typical of senescent cells. (B) Cells were seeded cells at 500, 1000, 5000, 10000, 20000, and 30000 cells per well and grown for 2 weeks at 34°C and 38°C and then stained with methylene blue; 226L 8/13 cells grew at the permissive temperature 34°C, but they arrested at the non-permissive temperature 38°C. (C) Crystal violet growth assays demonstrated that 226L 8/13 cells grew at 34°C, but at 38°C growth ceased. Error bars represent the standard error of the mean.

### **3.4 226L 8/13 cells stain for senescence associated $\beta$ -galactosidase when they undergo growth arrest at the non-permissive temperature**

One of the markers widely recognised for cellular senescence is senescence associated  $\beta$ -galactosidase, which is up-regulated upon senescence (Dimri et al., 1995). This assay is based on the finding that unlike pre-senescent, quiescent and immortal cells that only exhibit lysosomal hydrolase  $\beta$ -galactosidase activity at pH4.0, senescent cells to exhibit lysosomal hydrolase  $\beta$ -galactosidase activity at pH6.0 (Dimri et al., 1995).

226L 8/13 cells were grown at 34°C and 38°C for 2 weeks, and following this incubation period were stained for SA- $\beta$ -gal. To control for temperature shift, 226L U19/13 cells, which express a non-thermolabile LT antigen and therefore do not arrest at 38°C, were also incubated at 38°C and stained. Cells were then stained using the Sigma 'Senescence Cells Histochemical Staining Kit' and incubated at 37°C over night. Blue staining, indicative of SA- $\beta$ -gal activity, was clearly visible in 226L 8/13 cells grown at the non-permissive temperature (Figure 3.4, A). In the control 226L 8/13 cells grown at the 34°C and 226L U19/13 cells grown at 38°C, SA- $\beta$ -gal staining was not detected (Figure 3.4, B and C). This indicates that 226L 8/13 undergo senescence arrest at the non-permissive temperature.



**Figure 3.4: Arrested 226L 8/13 cells stain for SA-β- galactosidase**

226L 8/13 cells were grown at 34°C and 38°C for 2 weeks, and following this incubation period were stained for SA-β-gal. To control for temperature shift, 226L U19/13 cells, which express a non-thermolabile LT antigen and therefore do not arrest at 38°C, were also incubated at 38°C and stained. **(A)** Blue staining, indicative of SA-β-gal activity, was clearly visible in 226L 8/13 cells grown at the non-permissive temperature. **(B)** In the control 226L 8/13 cells grown at the 34°C and **(C)** 226L U19/13 cells grown at 38°C, SA-β-gal staining was not detected. This indicates that 226L 8/13 undergo senescence arrest at the non-permissive temperature.

### **3.5 Complementation in 226L 8/13 cells**

To immortalize breast luminal epithelial cells, Mike O'Hare found that hTERT was not sufficient but additional activities were required, which could be provided by SV40 LT antigen. SV40 LT inactivates the Rb and p53 tumour suppressors, both of which play a role in cellular senescence [reviewed by (Ben Porath and Weinberg, 2004)]. In a fibroblast system equivalent to the 226L 8/13 cells, the growth arrest appears to be very much dependent on the p53 pathway, only a small proportion of cells overcome the conditional growth arrest by inactivation of the pRb pathway (Rovillain et al., 2011). Genetic complementation of the 226L 8/13 cells by knock-down of genes involved in these pathways represents a strategy to identify the importance of the pRb and p53 pathways in cellular senescence in this epithelial system. Components of the p53 and pRb pathways were functionally abrogated in the 226L 8/13 cells to test if their abrogation could lead to cells overcoming the conditional growth arrest at the non-permissive temperature.

#### **3.5.1 Expression of wild-type large T antigen causes 226L 8/13 cells to overcome the conditional arrest - establishment of the 226L 8/13 complementation assay**

226L 8/13 cells arrest at the non-permissive temperature because the temperature-sensitive large T antigen is inactivated at this temperature. Therefore, reconstitution of large T activity at the non-permissive temperature by introduction of wild-type large T antigen into 226L 8/13 cells should lead to cells overcoming the conditional growth arrest.

10 $\mu$ g of pLPC-WTLT which expresses wild type LT (Rovillain et al., 2011) and pLPCX empty vector were packaged using  $\Phi$  amphotropic packaging cells. 226L 8/13 cells were seeded at 500,000 cells in T75 flasks and infected with 5 ml of viral supernatant in 15 ml full medium and 8  $\mu$ g/ml polybrene over night. The following day infection medium was replaced with full medium, and selection was commenced 48 hours post-infection with 2 $\mu$ g/ml puromycin. Cells were kept under selection for 5 days, after which period no viable cells remained in the non-infected control culture, whereas

multiple puromycin resistant clones were observed in infected cultures. After drug selection cells were reseeded at 80,000 cells in T75 flasks, which corresponds to 10,000 cells per well in 6-well plates (see Figure 3.3), and shifted to the non-permissive temperature the following day. Whereas a confluent monolayer was observed in the pLPC-WTLT-infected culture incubated at 38°C for 14 days, no outgrowing colonies were observed in the pLPCX control-infected culture incubated under the same conditions. The experiment was repeated three times and representative flasks are shown (Figure 3.5). This demonstrated that introduction of wild-type large T antigen was sufficient to overcome the conditional growth arrest at 38°C. Furthermore, this was used to define the conditions for the complementation assay (see methods section 2.1.7).

### **3.5.2 Abrogation of the p53 pathway**

To inactivate the p53 pathway two different reagents, GSE p53 and p53 shRNA, were used. GSE p53 functions as a dominant-negative peptide of p53 and was identified in a GSE screen. It corresponds to a region in the oligomerisation domain of p53 (Ossovskaya et al., 1996) and exerts its function as a dominant-negative peptide of p53 by promoting the accumulation of endogenous p53 protein into a functionally inactive form. There is however a high level of sequence conservation in the oligomerisation domain between p53 and the p53 family members p63 and p73 [reviewed by (Levrero et al., 2000)] therefore GSE p53 probably interacts with all three members of the p53 family. For this reason we also used p53 shRNA to abrogate p53 specifically.

#### ***3.5.2.1 Knock-down of p53 by shRNA complements the conditional growth arrest***

Louise Mansfield had previously shown that knock-down of p53 rescues HMF3A cells from the conditional growth arrest. She reconstructed an shRNA construct, pRetroSuper-p53, which had been shown to effectively knock down p53 (Berns et al., 2004). This construct was tested in the 226L 8/13 complementation assay, and pRetroSuper-LaminA/C (also made by Dr Louise Mansfield) was used as the negative control. Many colonies were observed in the p53 shRNA-infected culture incubated at

38°C for 14 days, whereas very few outgrowing colonies were observed in the lamin control-infected culture incubated under the same conditions. The experiment was repeated three times, and representative flasks are shown in Figure 3.5. This demonstrated that down-regulation of p53 by shRNA was sufficient to complement the growth arrest of these cells at the non-permissive temperature, but it was not as effective as reconstitution of SV40 LT antigen activity.

### ***3.5.2.2 Abrogation of p53 by p53 GSE complements the conditional growth arrest***

After growth complementation activity by p53 shRNA was confirmed, the ability of p53 GSE to complement growth under non-permissive conditions was tested. pLPCX and pLXIP-GSEp53 retroviral expression constructs were tested in the 226L 8/13 complementation assay. After 14 days of incubation at 38°C flasks were stained with methylene blue; multiple colonies were visible in the GSE p53-infected cultures, but very few colonies were observed in lamin control-infected cultures. This showed that abrogation of p53 with p53 GSE was sufficient to complement the 226L 8/13 cells' growth arrest at the non-permissive temperature. The complementation assay was repeated three times, and representative flasks are shown in Figure 3.5.

### ***3.5.2.3 Knock-down of p21 by shRNA complements the conditional growth arrest***

P21 is a downstream target of p53, and p21 knock-down by shRNA was sufficient to bypass the conditional growth arrest in two fibroblast cell lines analogous to the 226L 8/13 cells, namely BJ-TERT-tsLT (Berns et al., 2004) cells and HMF3A cells (Rovillain et al., 2011).

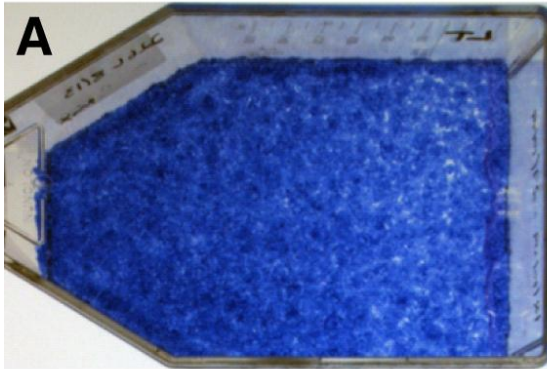
A pRetroSuper-p21 construct which expresses an shRNA that was shown to knock down p21 very effectively by Dr. Louise Mansfield was tested in the complementation assay. pRetroSuper-LaminA/C was used as a negative control. Following incubation at the non-permissive temperature, many growing colonies were observed in p21 shRNA-infected cultures, but not in the Lamin A/C shRNA-infected control cultures. The assay

was repeated three times, and representative flasks are shown in Figure 3.5. Levels of rescue achieved by knocking down p21 seemed slightly higher than levels of rescue achieved by knocking down p53, which had also been observed in the HMF3A cells (Rovillain et al., 2011). This might be due to more efficient knock-down by the p21 shRNA construct.

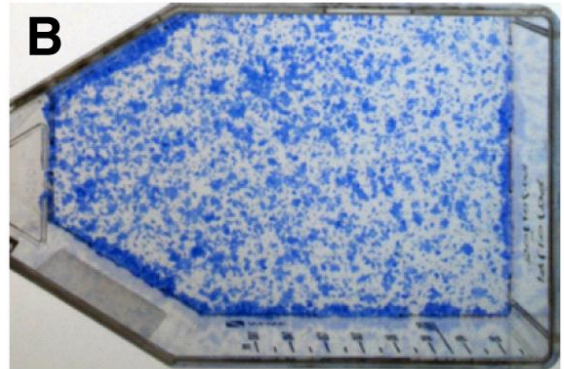
This demonstrated that knock-down of p21 achieved highly efficient abrogation of the 226L 8/13 conditional arrest.



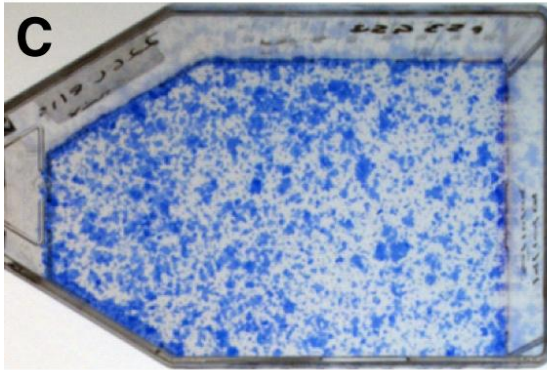
**wt LT antigen**



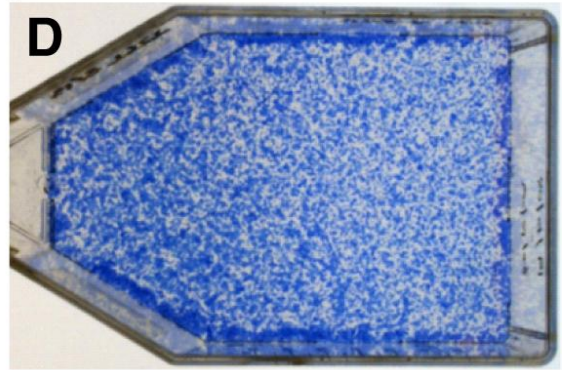
**p53 shRNA**



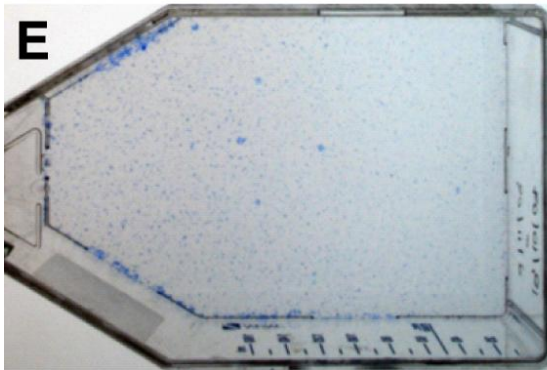
**p53 GSE**



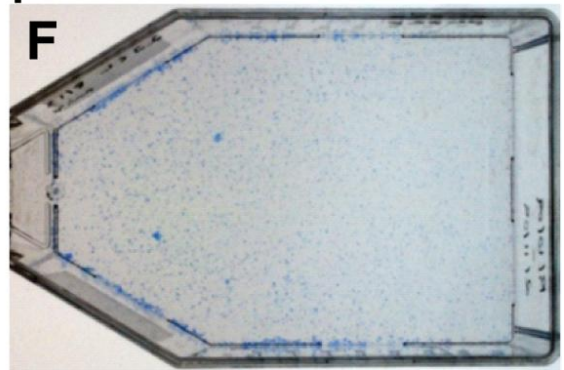
**p21 shRNA**



**Lamin**



**pLPCX**



**Figure 3.5: The p53 pathway and complementation**

226L 8/13 cells were infected with retroviral constructs expressing wt LT, p53 shRNA, p53 GSE, p21 shRNA and with the negative control lamin and empty pLPCX, and assayed for growth complementation. (A) expression of wt LT, silencing of p53 by (B) shRNA and (C) p53 GSE, and silencing of (D) p21 were sufficient to abrogate the conditional growth arrest of 226L 8/13 cells. Very low background levels were observed in negative controls (E) lamin and (F) pLPCX

### **3.6 Abrogation of the pRb pathway**

The viral oncoproteins adenovirus type 5 E1A 12S and HPV type 16 E7, both of which are known to bind and sequester the RB family of proteins (Dyson et al., 1992; Dyson, 1998; Dyson et al., 1989b; Whyte et al., 1988), are two reagents commonly used to inactivate this pathway. Therefore, the effect of ectopic expression of E1A and E7 on the conditional arrest of 226L 8/13 cells was tested.

#### **3.6.1 Constitutive Expression of Adenovirus Type 5 E1A complements the conditional growth arrest**

An E1A retroviral expression construct, pLPC-12SE1AORI (gift from S. Lowe) (Rovillain et al., 2011), was tested in the 226L 8/13 complementation assay to establish if it is sufficient to abrogate the conditional growth arrest. PLPCX was used as a negative control. Following incubation at the non-permissive temperature for 2 weeks multiple growing colonies were observed in E1A-infected cultures, but not in control pLPCX-infected cultures. The complementation assay was repeated three times, and representative flasks are shown in Figure 3.6. The rescue achieved by E1A was significantly lower than rescue achieved by p53 shRNA, GSEp53, and p21 shRNA. Furthermore, a lot of dead cells were observed in the culture medium, which is consistent with the finding that over-expression of E1A can induce apoptosis (Mymryk et al., 1994; Rao et al., 1992). E1A infection changed the morphology of 226L 8/13 cells, it amplified the cobblestone-like appearance (cells became more cuboidal and grew very tightly packed) and reduced size of the cells.

#### **3.6.2 Constitutive Expression of HPV Type 16 E7 is not sufficient to complement the conditional growth arrest**

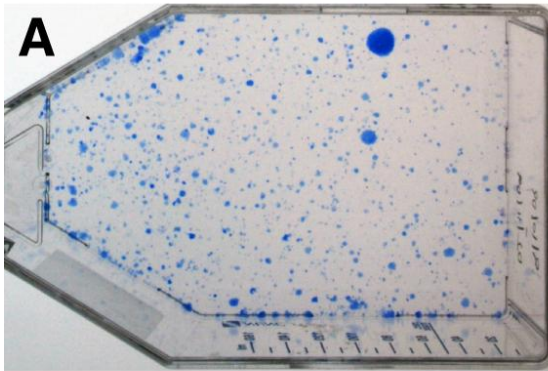
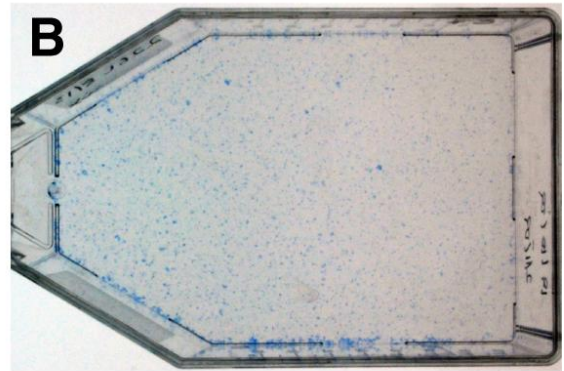
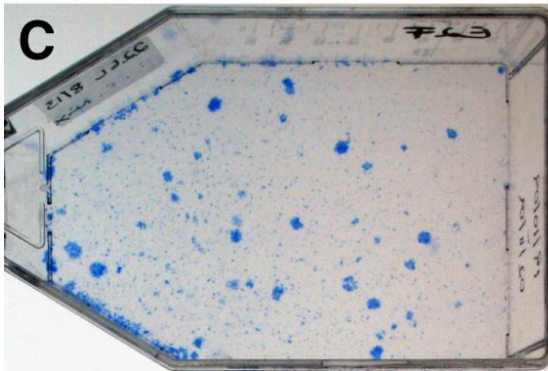
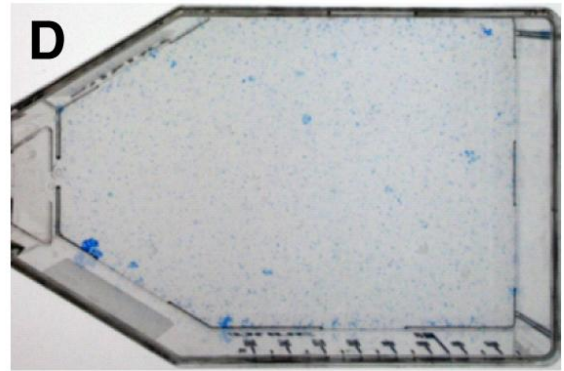
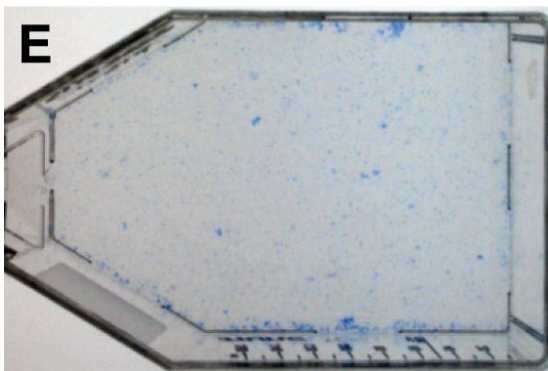
E7, like LT and E1A, functionally inactivates Rb family members by interaction via the the LXCXE-binding motif. An E7 retroviral expression construct, pBabepuro-E7 (gift from K. Munger) (Rovillain et al., 2011), was tested in the complementation assay for its ability to bypass the 226L 8/13 cell conditional growth arrest at the non-permissive temperature. pBabepuro was used as the negative control. After 2 weeks of incubation at the non-permissive temperature, no growing colonies were observed on either the control pBabepuro-infected cells, or the pBabepuro-E7 infected cells. The experiment was repeated three times, and representative flasks are shown in Figure 3.6. This result

is in contrast to HMF3A cells, in which E7 has been shown to overcome the conditional growth arrest (Rovillain et al., 2011).

### **3.6.3 Constitutive ectopic expression of the non-pRb binding E2F mutant E2F-DB complements the conditional growth arrest**

The best known targets of pRb are the E2F transcription factors (Bagchi et al., 1991; Bandara and Lathangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991). Zhang and colleagues (Zhang et al., 1999a) found that expression of E2F-DB, a mutant E2F-1 which lacks the C-terminal transactivation domain and the pRb binding domain but can still bind to DNA, could overcome p16<sup>INK4A</sup>, TGFβ and contact inhibition induced growth arrest. This suggests that a major role of E2F in cell cycle control is to form an active repressor complex with pRb at promoters to inhibit transcription required for cell cycle progression in addition to its role of activating transcription required for cell cycle progression, as E2F-DB lacks the transactivation domain and can therefore not exert its effect via activation of transcription. Rather, by competing for promoter binding sites with E2F/pRb repressive complexes, it can inhibit E2F/pRb mediated repression of transcription.

An E2F-DB retroviral expression construct was constructed by Dr. Louise Mansfield by cloning the E2F-DB open reading frame (gift from Pr. Xin Lu) into the pLPCX vector (Rovillain et al., 2011). pLPCX was used as the negative control. Following 2 weeks of incubation at the non-permissive temperature multiple colonies were observed in the pLPC-E2F-DB-infected cultures, but very low background levels were observed in control pLPCX-infected cultures. The complementation assay was repeated three times, and representative flasks are shown in Figure 3.6. This demonstrated that the 226L 8/13 cell conditional growth arrest can be abrogated by inhibition of the transcriptional repression activity of the Rb-E2F repressor complex, which is consistent with results observed in the HMF3A cells (Rovillain et al., 2011). Abrogation of the growth arrest by E2F-DB was less efficient than by p53 and p21 inhibition, and also slightly less efficient than abrogation of the growth arrest achieved by E1A. Even though colonies in E2F-DB infected cells grew to a slightly larger size, many more colonies were observed in E1A infected cells.

**E1A****E7****E2F-DB****pLPCX****pBabepuro****Figure 3.6: The pRb pathway and complementation**

226L 8/13 cells were infected with retroviral constructs expressing E1A, E7, E2F-DB and with the negative control empty vectors pBabepuro and pLPCX, and assayed for growth complementation. (A) expression of E1A was sufficient, (B) E7 was insufficient, and (C) E2F-DB was sufficient to abrogate the conditional growth arrest of 226L 8/13 cells. Very low background levels were observed in negative controls (D) pLPCX and (E) pBabepuro

## 3.7 Discussion

### 3.7.1 226L 8/13 cells express the epithelial markers $\beta$ -catenin and E-cadherin

226L 8/13 cells had been developed as a model to study cellular senescence in epithelial cells. They were derived from luminal epithelial cells. To confirm that 226L 8/13 cells had not lost their epithelial phenotype due to culturing conditions or the immortalization process they were stained for two epithelial markers, namely  $\beta$ -catenin and E-cadherin, and it was demonstrated that they express both markers (Figure 3.1).  $\beta$ -catenin was observed mainly in the membrane at cell-to-cell boundaries, and this correlates with the fact that it plays a crucial role in cell–cell adhesion. It has been shown that normal epithelia display cell boundary staining for beta-catenin (Hao et al., 1997). In addition to cell boundary staining, some cytoplasmic staining was also observed. It has been shown that cytoplasmic localization of beta-catenin is associated with poor outcome in breast cancer patients, but some cytoplasmic staining is normal (Lopez-Knowles et al., 2010).

E-cadherin staining was observed in the cytoplasmic membrane of 226L 8/13 cells, which is consistent with it being a transmembrane protein, and with the finding that it is strongly expressed in the cytoplasmic membrane of normal ductal epithelial cells in the mammary gland (Bukholm et al., 1998; Bukholm et al., 2000). Some punctate staining was observed with the E-cadherin antibody. This was also observed in the negative control HMF3A fibroblasts. The punctate staining was not observed in the secondary antibody alone control (data not shown), furthermore the same secondary antibody was used for  $\beta$ -catenin staining where the punctate staining was not observed; therefore it was most likely not an artefact caused by the secondary antibody. The punctate staining could represent E-cadherin being internalized. Le and colleagues observed punctate cytoplasmic E-cadherin staining, and they demonstrated that this represents cell surface E-cadherin being actively internalized and then recycled back to the plasma membrane (Le et al., 1999). They showed that in cells without stable cell-cell contacts the amount of E-cadherin undergoing endocytosis and recycling was increased. Le and colleagues suggest that surface trafficking of E-cadherin through an endocytic recycling pathway may provide a mechanism for regulating the availability of E-cadherin for junction



formation in development, tissue remodelling, and tumourigenesis. Furthermore, punctate membrane or cytoplasmic E-cadherin staining has been observed in some tumour cells (Harigopal et al., 2005). Immortalisation and cell culture conditions could have led to increased levels of internalised E-cadherin, but to confirm this, the primary luminal epithelial cells from which 226L 8/13 cells were derived would need to be stained for E-cadherin. Interestingly, the punctate staining pattern was also observed in the HMF3A fibroblasts. Generally, fibroblasts do not express E-cadherin, and no membrane staining for E-cadherin was observed in HMF3A cells; therefore the punctate staining is unlikely to represent internalised E-cadherin. In conclusion, it is most likely that the punctate staining was an artefact caused by the primary antibody or staining procedure.

Most cancers are derived from epithelial cells, and overcoming finite proliferative potential, i.e. senescence, is required for the development of cancer (Hanahan and Weinberg, 2011). Many studies are done in fibroblasts, but cancer is cell type specific, and the fact that 226L 8/13 cells are epithelial makes them a highly relevant model to study senescence.

### **3.7.2 226L 8/13 cells express luminal and basal markers**

226L 8/13 cells were positive for the luminal marker cytokeratin 18 and the basal markers cytokeratin 14 and  $\beta$ -4 integrin, and they were negative for the luminal marker cytokeratin 19 (Figure 3.2).

Originally, luminal cells were isolated to generate the 226L 8/13 cells, but culturing the cells *in vitro* and immortalisation could have caused expression of such markers to change (Moll et al., 1982). This could have caused cells to express the basal markers cytokeratin 14 and  $\beta$ -4 integrin and lose expression of the luminal marker cytokeratin 19. Unfortunately earlier cells were not available for analysis of luminal and basal marker expression. It has been reported that cytokeratin 19 expression can be lost as a consequence of immortalization (Spancake et al., 1999), however others have shown that this is not necessarily the case (Gudjonsson et al., 2002; Wazer et al., 1995). It is possible that the lack of cytokeratin 19 expression was due to immortalization of a cell from the cytokeratin negative subpopulation of cells, since it has been suggested that in the normal mammary gland, cytokeratin 19 is expressed in only a subpopulation of

luminal epithelial cells within the terminal ductal lobular unit (TDLU) (Bartek et al., 1985). 226L 8/13 cells were positive for the basal marker cytokeratin 14. Bartek and colleagues found that when they immortalized breast luminal epithelial cells with SV40 LT antigen about 30% of the colonies expressed cytokeratin 14 (Bartek et al., 1991), which has been shown to be induced in some luminal cells when grown in culture (Taylor-Papadimitriou et al., 1989). Furthermore, even though generally only basal cells express cytokeratin 14, it has been shown that some keratin 14 expression is seen in luminal cells in the very large ducts (O'Hare et al., 1991; Taylor-Papadimitriou et al., 1989).

During the immortalisation process, 226L 8/13 cells were selected against  $\beta$ -4 integrin at every clonal level (up to clone level 8); therefore it is surprising that they express  $\beta$ -4 integrin. It is possible that cell culture conditions put strong pressure on them to express  $\beta$ -4 integrin. Also, as negative selection by the antibody was not confirmed at each selection step, it is possible that the sorting was unsuccessful. Nonetheless, since 226L 8/13 cells were initially derived from luminal epithelial cells, immortalisation and/or culturing the cells seems to have induced expression of this basal marker.

### **3.7.3 226L 8/13 cells grow at 34°C, and they enter a senescence arrest at 38°C**

It was demonstrated that 226L 8/13 cells grow at 34°C, at which temperature the temperature-sensitive LT is active, and that they cease to grow at 38°C, where the ts LT is inactive (Figure 3.3). Cells were stained for senescence associated  $\beta$ -galactosidase, a widely recognised marker of cellular senescence (Dimri et al., 1995). 226L 8/13 cells incubated at the non-permissive temperature for two weeks were positive for this marker, whereas 226L 8/13 cells grown at the permissive temperature were negative (Figure 3.4), supporting that the arrest at 38°C was senescence arrest. However, the use of SA  $\beta$ -galactosidase as a senescence biomarker remains controversial; for example, positive SA  $\beta$ -galactosidase activity may also occur under senescence-independent conditions such as stressful culture conditions (Iwasa et al., 2003) or confluence (Gary and Kindell, 2005). Other markers of cellular senescence could be used to confirm that the growth arrest at the permissive temperature is senescence, such as SAHFs (Narita et

al., 2003), senescence-associated DNA-damage foci (SDFs) which can be caused by dysfunctional telomeres or other sources of DNA damage [reviewed by (Campisi and d'Adda di Fagagna, 2007)] and 'DNA segments with chromatin alterations reinforcing senescence' (DNA-SCARS) (Rodier et al., 2011). Although DNA-SCARS are novel markers of senescence in normal cells, they can only be used as senescence markers in combination with other markers, because DNA-SCARS are associated with, but not exclusive to, senescent cells. Particularly, p53-deficient cells spontaneously develop DNA-SCARS, but they proliferate.

#### **3.7.4 Complementation – relative importance of the p53 and pRb pathway**

First, it was demonstrated that reconstitution of wt LT activity by ectopic expression of wt LT was sufficient to overcome the conditional growth arrest of 226L 8/13 cells. This indicated that the 226L 8/13 system could be used to dissect the pathways underlying the cellular senescence in epithelial cells by genetic complementation. A 226L 8/13 complementation assay was developed and used to determine the relative importance of the p53 and pRb pathways in the loss of growth potential upon inactivation of LT. It was shown that the 226L 8/13 conditional growth arrest could be complemented at the non-permissive temperature by inhibition of the p53 pathway by expressing GSE p53 or shRNAs against p53 and p21 (Figure 3.5). Growth complementation by inhibition of the p53 pathway was highly efficient, even though slightly less efficient than reconstitution of LT activity. Furthermore, it was shown that inhibition of the pRb pathway by expression of E1A and E2F-DB could overcome the conditional growth arrest of 226L 8/13 cells (Figure 3.6), but this was far less efficient than inhibition of the p53 pathway. Expression of E1A and E2F-DB only caused a proportion of cells to overcome the growth arrest. Inhibition of the pRb pathway by expression of E7 was insufficient to overcome the conditional arrest.

It was not surprising that inhibition of the p53 pathway was more efficient than inhibition of the pRb pathway, as this had also been observed in HMF3A cells, which are the fibroblast counterpart of the 226L 8/13 cells (Rovillain et al., 2011). However, the finding that inhibition of the p53 pathway did not abrogate the growth arrest as



efficiently as expression of wt LT, which inhibits both the p53 and pRb pathway, suggests that pRb provides an additional barrier to stop these cells from proliferating.

It was surprising that E7 was not sufficient to abrogate the conditional arrest, which was in contrast to HMF3A cells, in which it has been shown to be sufficient to abrogate the conditional arrest (Rovillain et al., 2011). It is possible that this was due to cell type specific differences; E7 might be less efficient in epithelial cells. On the other hand, expression of E7 in the 226L 8/13 cells was never demonstrated, and it is therefore possible that it was simply not expressed in these cells. E7 was introduced with the construct pBabepuro-E7, which expresses E7 under control of the LTR (puromycin resistance is under control of the SV40 promoter), whereas all the other constructs' expression cassettes were under control of the CMV promoter. The LTR is not as strong as CMV, and might not have led to efficient expression of E7 in the 226L 8/13 cells, thereby causing the E7 construct not to have an effect in these cells. Because E7 and puromycin resistance are under the control of different promoters in pBabepuro-E7 it is possible that puromycin resistance was expressed more efficiently than E7. This would allow cells to survive drug selection without expressing E7.

### **3.7.5 What causes the 226L 8/13 cells to arrest at 38°C**

Because the 226L 8/13 cells express hTERT, the senescence arrest at the non-permissive temperature cannot be caused by telomere shortening. It is unclear what causes the cells to arrest upon inactivation of ts LT at the non-permissive temperature.

Rodier (Rodier et al., 2011) and colleagues showed the presence of DNA-SCARS in senescent cells, which are relatively stable DNA-damage foci that are distinct from transient damage foci, and they demonstrated that SCARS are important for the senescence arrest. DNA-SCARS are associated with, but not exclusive to, senescent cells. Particularly, p53-deficient cells spontaneously develop DNA-SCARS, but they proliferate. Rodier and colleagues showed that cells expressing SV40LT harboured senescence levels of DNA-SCARS but continued to proliferate, indicating that defective cell cycle checkpoints can uncouple DNA-SCARS from the senescence growth arrest. SCARS initiate senescence via DNA-damage response (DDR) signalling.

226L 8/13 cells express SV40LT, which inhibits both the pRb and the p53 pathway. It is therefore possible that the cells harbour SCARS when grown at the permissive temperature and that when SV40 LT is inactivated at the non-permissive temperature, p53 and pRb become active and induce senescence caused by SCARS. 226L 8/13 cells grown at both 34°C and 38°C should be tested for the presence of DNA-SCARS. This leads to the question of why both hTERT and SV40LT were required to immortalize human breast luminal epithelial cells, whereas other groups have shown that introduction of hTERT alone is sufficient to immortalize several somatic cell types (Bodnar et al., 1998; Ouellette et al., 2000; Vaziri and Benchimol, 1998; Yang et al., 1999). Possibly, forcing these cells to divide *in vitro* could lead to the development of DNA-SCARS, leading to cells senescing in the presence of a functional p53 and pRb pathway; this might explain the requirement of inhibition of the pRb and p53 pathway in addition to introduction of hTERT. It has been suggested that when cells are cultured under adequate conditions hTERT is sufficient to immortalize; Herbert and colleagues found that primary HMECs can be immortalized by introduction of hTERT without abrogating the pRb/p16<sup>INK4a</sup> and p53 pathway (Herbert et al., 2002). It would be interesting to test primary luminal epithelial cells whose life-span has been extended by the introduction of telomerase for the presence of DNA-SCARS.

The fact that the p53 pathway plays an important role in the induction of senescence in 226L 8/13 cells suggests that the arrest might be induced by DDR. The p53 pathway has been shown to play an important role in DNA-damage induced senescence (d'Adda di Fagagna et al., 2003; DiLeonardo et al., 1994; Herbig et al., 2004). When telomeres become critically short they trigger senescence via initiation of the DDR (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Takai et al., 2003), which induces senescence via DNA damage-induced p53 signalling pathway [reviewed by (Deng et al., 2008)]. But p16<sup>INK4a</sup> has also been shown to be induced in response to DNA damage and dysfunctional telomeres, and it is believed to function as an additional barrier to stop cells with damaged DNA or telomeres from re-entering the cell cycle (Beausejour et al., 2003; Jacobs and de Lange, 2004). P16<sup>INK4a</sup> knock-down alone was not sufficient to overcome a TRF2<sup>ΔBΔM</sup>-induced telomere-induced senescence arrest, but p16<sup>INK4a</sup> did seem to contribute to the proliferation arrest as inhibition of p53 led to a partial escape from TRF2<sup>ΔBΔM</sup>-induced growth arrest, whereas inhibition of both p16<sup>INK4a</sup> and p53

together lead to a near complete restoration of the rate of DNA synthesis and an increased rescue from growth arrest (Jacobs and de Lange, 2004). This also supports the hypothesis that 226L 8/13 senescence arrest is caused by DNA damage, as the pRb pathway seems to play a minor role in these cells. It should be tested if inhibition of the pRb pathway in addition to the p53 pathway would increase abrogation of the growth arrest of 226L 8/13 cells.

## **4 Results – Establishment and Characterisation of the sub-clone, EcoR Clone 7**

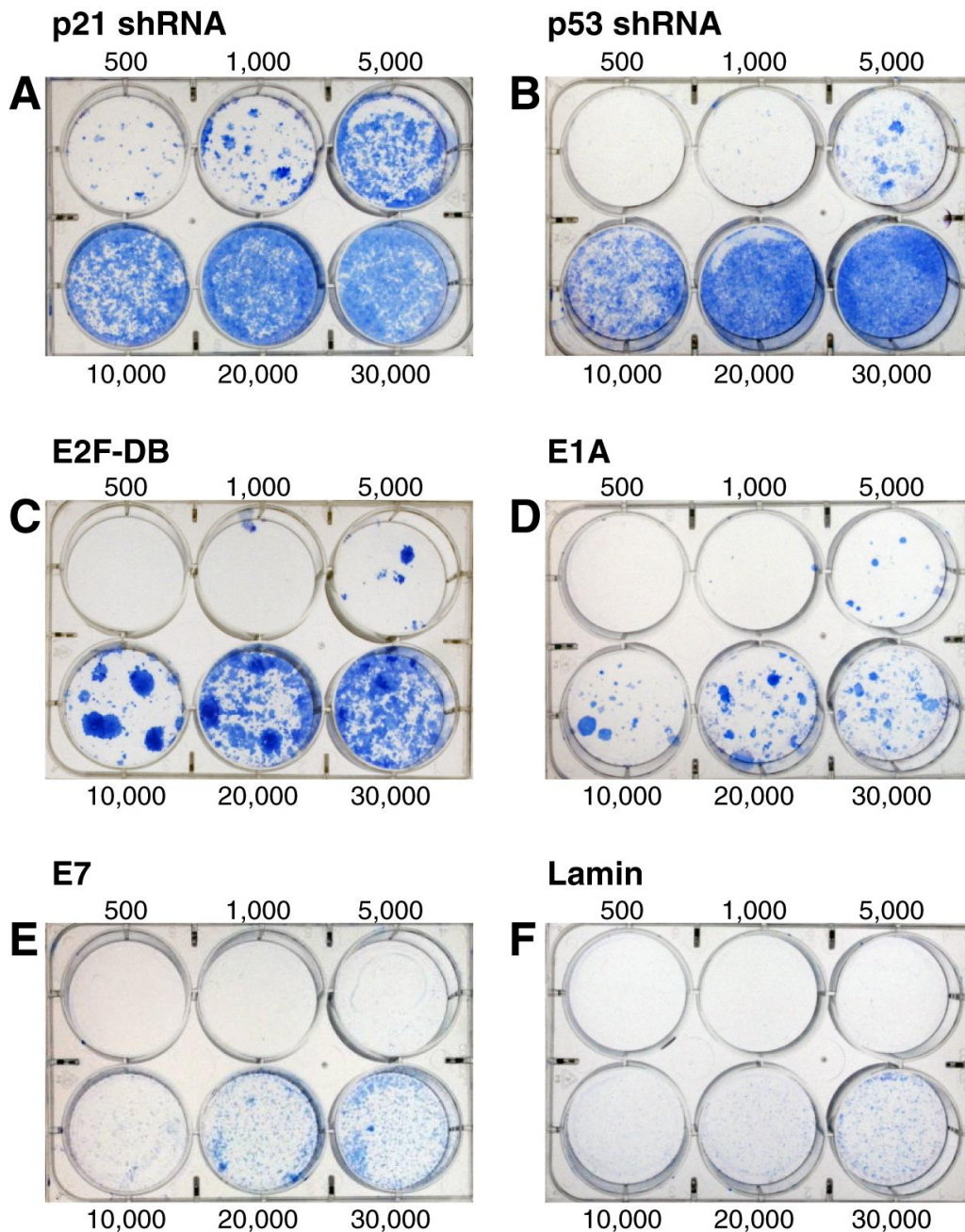
### **4.1 Introduction of the ecotropic receptor into 226L 8/13 cells**

Human cells have previously been engineered to express the murine ecotropic receptor to make them infectable with ecotropic retroviruses [for example (Berns et al., 2004)]. The use of ecotropic viruses increases safety, as human cells cannot be infected by such viruses. Furthermore, much higher viral titers can be achieved with ecotropic compared to amphotropic  $\Phi$  packaging cells. Therefore, the murine ecotropic receptor cDNA sequence (EcoR; gift from J. Downward) which had been cloned into pWZLBlast $\epsilon$  by Dr. Louise Mansfield was introduced into the 226L 8/13 cells. 10 $\mu$ g of pWZLBlast $\epsilon$ -EcoR were packaged using  $\Phi$  amphotropic packaging cells and 250 $\mu$ l, 500 $\mu$ l, 1ml and 2ml of the viral supernatant was used to infect 226L 8/13 cells seeded at 500,000 cells in T75 flasks over night. 48 hours post-infection 5 $\mu$ g/ml blasticidin was added to the culture medium and after completion of 10 days of drug selection no viable cells remained in the non-infected control culture, whereas multiple blasticidin resistant clones were observed in all infected 226L 8/13 cultures. To obtain uniform cultures single cell clones were picked; this was of importance as for the planned shRNA screen cells needed to stably express the ecotropic receptor, be highly infectable with ecotropic virus, and also show high conditionality for growth at the permissive temperature and the ability to be rescued from the conditional growth arrest by inhibition of the p53 and pRb pathways. In the culture infected with 500 $\mu$ l virus single cell clones were well separated, which enabled isolation of single clones. Drug resistant clones were ring-cloned and grown in the presence of blasticidin for a further 14 days. Stable expression of the ecotropic receptor was demonstrated by the presence of puromycin resistant colonies following infection of the 226L 8/13 EcoR clones with ecotropic retroviruses conferring puromycin drug resistance (see below).

### **4.2 Clone screening to identify a clone suitable for the screen**

To identify a clone for the screen, several clones isolated after transduction with the ecotropic receptor were screened for complementation with p53 shRNA, p21 shRNA,

E7, E1A and E2F-DB. 10 $\mu$ g of pRetroSuper-p53, pRetroSuper-p21, pLPCX-12SE1AORI, pBabepuro-E7, pLPC-E2F-DB, and pRetroSuper-LaminA/C were packaged using  $\Phi$  ecotropic packaging cells, and 226L 8/13 EcoR clones were seeded at 500,000 cells in T75 flasks and infected with 5ml viral supernatant over night. 48 hours post-infection 2 $\mu$ g/ml puromycin was added to the culture medium and after completion of 5 days of drug treatment, no viable cells remained in the non-infected culture, whereas multiple puromycin-resistant clones were observed in all infected cultures. Puromycin was removed from the culture medium and drug-resistant clones were reseeded at 500, 1,000, 5,000, 10,000, 20,000 and 30,000 cells/well in 6 well plates and shifted to 38°C. 10,000 cells corresponds to approximately 80,000 cells in T75 flasks, which was used in the complementation assay with parental 226L 8/13 cells (Figures 3.5 and 3.6). Cells were then grown for 2 weeks before they were stained with methylene blue. Several 226L 8/13 EcoR clones fulfilled the above stated requirements, but 226L 8/13 EcoR Clone 7 exceeded all the other clones in fulfilling these criteria. When infected with the negative control pRetroSuper-LaminA/C background levels of growth at the non-permissive temperature were very low (Figure 4.1 F), whereas efficient complementation was achieved with p53 shRNA (Figure 4.1 B) and p21 shRNA (Figure 4.1 A). Complementation was also achieved with E1A (Figure 4.1 D) and with E2F-DB (Figure 4.1 C), but like in the parental 226L 8/13 cells, E7 was not sufficient to complement the conditional growth arrest (Figure 4.1 E). 226L 8/13 EcoR Clone 7 mirrored the parental cells and were likely to represent a good candidate for the large-scale shRNAmir screen.



**Figure 4.1: EcoR Clone 7 complementation assay**

EcoR Clone 7 cells were infected with retroviral constructs expressing p53 shRNA, p21 shRNA, E1A, E7, E2F-DB and with the negative control lamin. Following drug selection cells were seeded at 500, 1000, 5000, 10000, 20000, and 30000 cells and tested in the complementation assay. (A) p21 shRNA, (B) p53 shRNA, (C) E2F-DB and (D) E1A were sufficient to bypass the conditional arrest. (E) E7 was insufficient to abrogate the growth arrest. (F) Very low background levels were observed in negative control-infected cells

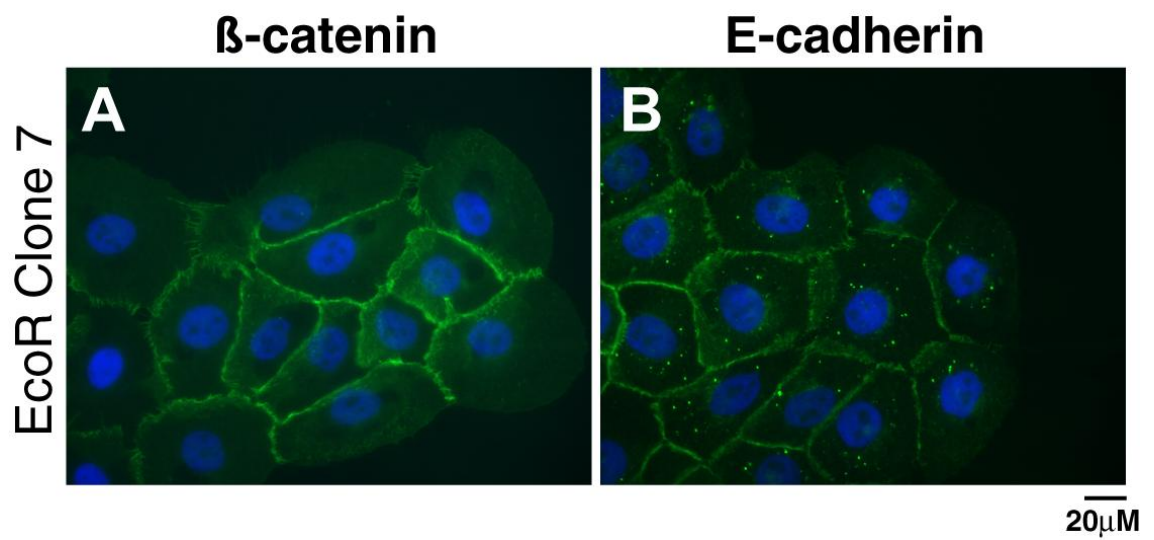
### **4.3 Characterisation of EcoR Clone 7**

226L 8/13 EcoR Clone 7 was a promising candidate for the screen based on the complementation assay. But to confirm that 226L 8/13 EcoR Clone 7 was suitable, it needed to be characterized to ensure its phenotypic characteristics did not deviate too far from the parental 226L 8/13 cells.

#### **4.3.1 EcoR Clone 7 cells express the epithelial markers $\beta$ -catenin and E-cadherin**

226L 8/13 EcoR Clone 7 cells were seeded at 10,000 cells on coverslips in 24-well plates. When they reached about 60% confluence they were fixed and stained for the epithelial markers  $\beta$ -catenin and E-cadherin. Nuclei were stained with Dapi. Like the parental 226L 8/13 cells, cells stained positive for both markers, confirming their epithelial phenotype (Figure 4.2). EcoR Clone 7 cells were stained with the parental cells, therefore the same controls are shown: Negative control cells, HMF3A mammary fibroblasts, were negative for both markers (Figure 3.1, C and D). Secondary antibody alone controls did not show any staining (data not shown). As in parental 226L 8/13 cells,  $\beta$ -catenin was observed mainly on the membrane at cell-to-cell boundaries, which correlates with the finding that normal epithelia display cell boundary staining for beta-catenin (Hao et al., 1997). Some cytoplasmic staining was also observed. The transmembrane glycoprotein E-cadherin was observed in the plasma membrane as expected. As previously observed in the parental 226L 8/13 cells, some punctate staining was observed with the E-cadherin staining.

These results showed that like 226L 8/13 cells, EcoR Clone 7 cells have retained their normal epithelial phenotype and therefore represent a good model system to study epithelial cells.



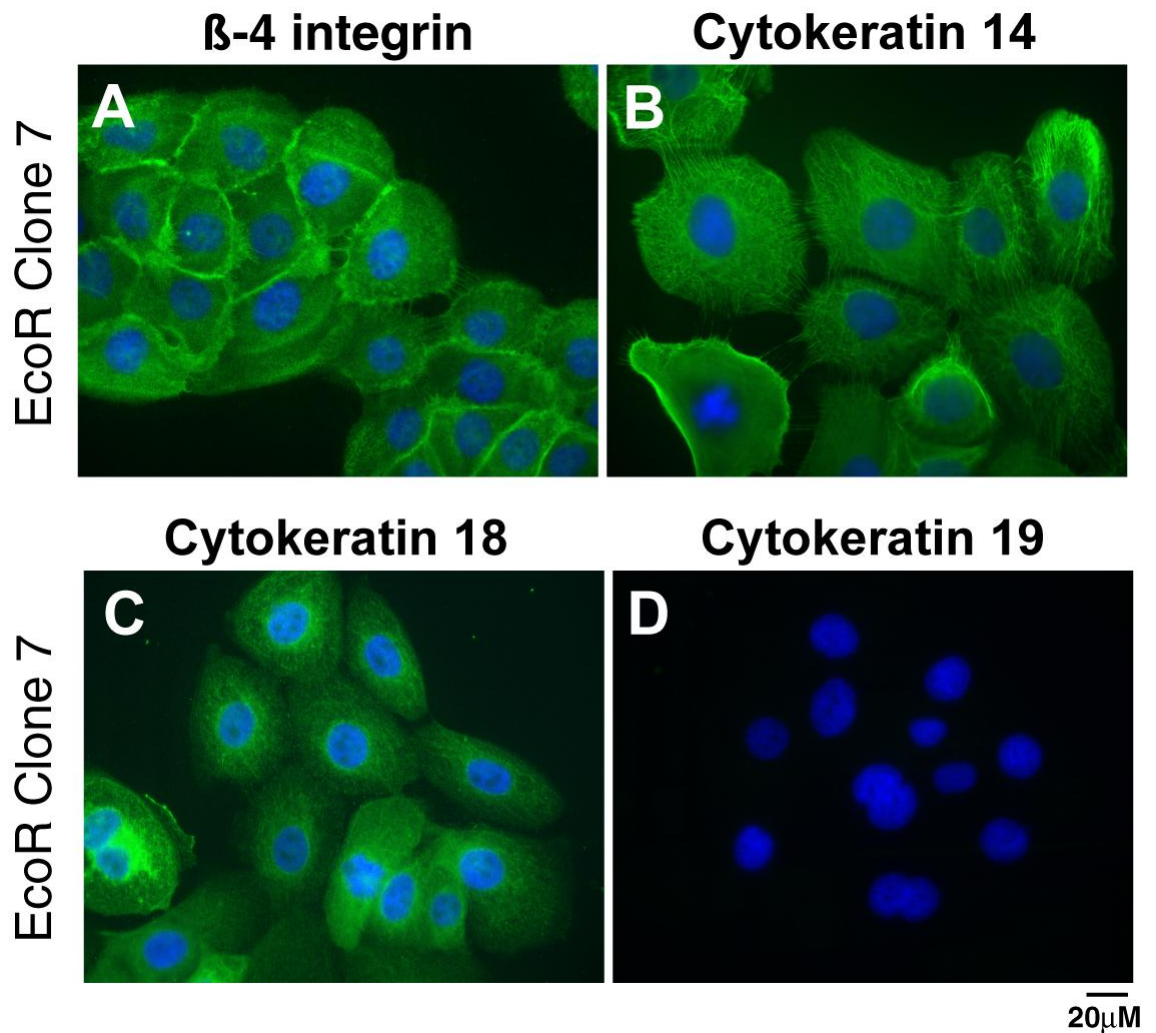
**Figure 4.2: EcoR Clone 7 cells express the epithelial markers  $\beta$ -catenin and E-cadherin**

EcoR Clone 7 cells were stained for the epithelial markers  $\beta$ -catenin and E-cadherin. **(A)** EcoR Clone 7 cells express  $\beta$ -catenin. **(B)** EcoR Clone 7 cells express E-cadherin.



#### **4.3.2 EcoR Clone7 cells express luminal and basal markers**

226L 8/13 EcoR Clone 7 cells were stained for the luminal markers cytokeratin 18 and cytokeratin 19, and for the basal markers cytokeratin 14 and  $\beta$ -4 integrin. Like the parental 226L 8/13 cells, cells stained positive for both basal markers (Figure 4.3, A and B), and the luminal marker cytokeratin 18 (Figure 4.3, C), but were negative for the luminal marker cytokeratin 19 (Figure 4.3 D). The intermediate filaments were visible in cytokeratin 14 and 18 staining and both had their typical appearance (O'Hare et al., 1991), and  $\beta$ -4 integrin staining also had its typical appearance. EcoR Clone 7 cells were stained together with parental cells, therefore the same controls are presented: HMF3A mammary fibroblasts were used as negative controls, and they were negative for all 4 markers (Figure 3.2b, A, B and C). HB4A cells, which have been shown to express cytokeratin 19 (O'Hare et al., 1991), were used as a positive control for cytokeratin 19 staining; HB4A cells stained positive for cytokeratin 19, confirming that the staining protocol and antibody worked (Figure 3.2b, D).



**Figure 4.3: EcoR Clone7 cells express both luminal and basal markers**

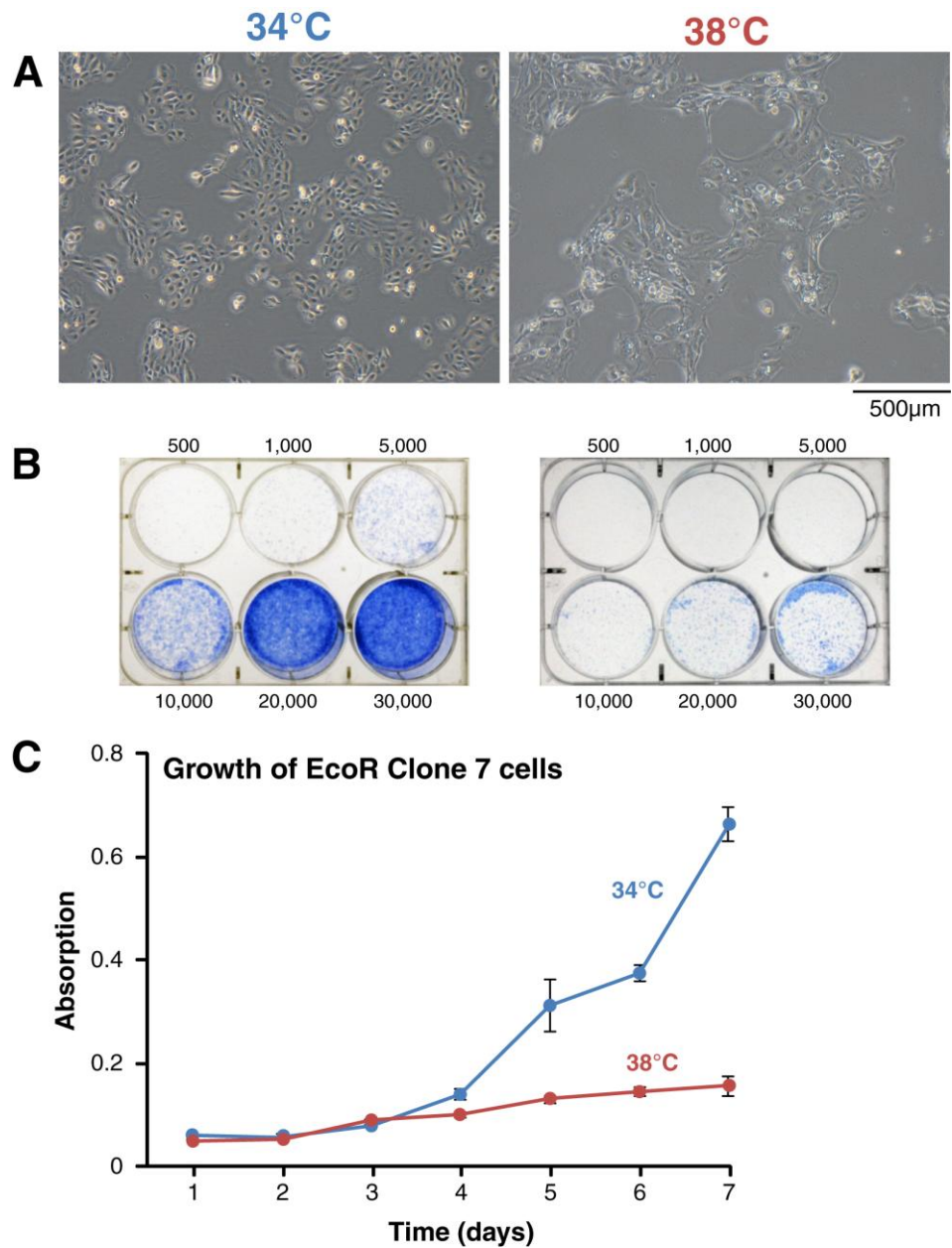
EcoR Clone 7 cells were stained for the basal markers  $\beta$ -4 integrin and cytokeratin 14, and for the luminal markers cytokeratin 18 and cytokeratin 19. They express (A)  $\beta$ -4 integrin, (B) cytokeratin 14 and (C) cytokeratin 18. (D) EcoR Clone 7 cells do not express cytokeratin 19.

#### **4.3.3 EcoR Clone 7 Cells grow at the permissive temperature, 34°C, but they cease to grow at the non-permissive temperature, 38°C**

To determine the ability of the EcoR Clone 7 cells to proliferate at 34°C and 38°C, cells were seeded and incubated at 34°C and 38°C for 7 days. Whereas cultures grown at the permissive temperature had to be split once, cultures at the non-permissive temperature ceased to grow and did not need to be split. After 7 days at each temperature cultures were photographed. Representative images are shown in Figure 4.4 A. Like the parental 226L 8/13 cells, they grew in islands, which is typical of epithelial cells, and had the characteristic cobblestone morphology of epithelial cells. EcoR Clone 7 cells incubated at the non-permissive temperature acquired a phenotype typical of senescent cells; they were enlarged with a flattened morphology and vacuole rich cytoplasm.

To confirm that EcoR Clone 7 cells grow at 34°C but arrest at 38°C, cells were seeded at 500, 1000, 5000, 10000, 20000, and 30000 cells in 6 well plates and grown for 2 weeks at the permissive and at the non-permissive temperature. Representative images are shown in Figure 4.4 B. Growing colonies were clearly visible on the plate incubated at 34°C, but apart from very little staining at the highest cell density (30,000 cells) no blue colonies were visible on the plate incubated at 38°C. This confirmed that EcoR Clone 7 cells grow at the permissive temperature but arrest at the non-permissive temperature.

Furthermore, direct growth assays were performed to confirm that EcoR Clone 7 cells grow at 34°C but arrest at 38°C. 500 cells per well were plated in 96 well plates, and cells were incubated at 34°C and at 38°C. 3 wells for each temperature were stained every day from day 1 (24 hours after cells were seeded) until day 7. Plates were fixed and stained with crystal violet, and optical density was measured at 590 nm in a luminometer. At 38°C cells grew at approximately the same rate as at 34°C until day 3, when they started slowing down and growth ceased (Figure 4.4 C). This confirms that 226L 8/13 EcoR Clone 7 cells grow at the permissive temperature, but they cease to grow at the non-permissive temperature.

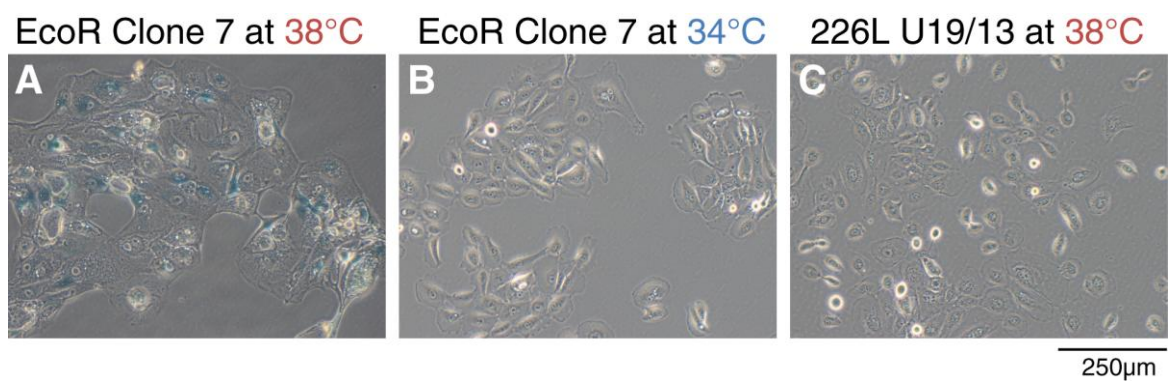


**Figure 4.4: Growth of EcoR Clone 7 cells at 34°C and 38°C**

(A) EcoR Clone 7 cells incubated at the permissive temperature showed the normal morphology of dividing epithelial cells. EcoR Clone 7 cells incubated at the non-permissive temperature acquired a phenotype typical of senescent cells. (B) Cells were seeded cells at 500, 1000, 5000, 10000, 20000, and 30000 cells per well and grown for 2 weeks at 34°C and 38°C and then stained with methylene blue; EcoR Clone 7 cells grew at the permissive temperature 34°C, but they arrested at the non-permissive temperature 38°C. (C) Crystal violet growth assays demonstrated that EcoR Clone 7 cells grew at 34°C, but at 38°C cells ceased to grow. Error bars represent the standard error of the mean.

#### **4.3.4 EcoR Clone 7 cells stain for senescence associated beta galactosidase when they undergo growth arrest at the non-permissive temperature**

To assess whether EcoR Clone 7 cells senesce at the non-permissive temperature, they were tested for expression for SA  $\beta$ -galactosidase. EcoR Clone 7 cells were grown at 34°C and 38°C for 2 weeks, and following this incubation period were stained for SA- $\beta$ -gal. To control for effects of the temperature shift, 226L U19/13 cells, which express a non-thermolabile LT antigen and therefore do not arrest at 38°C, were also incubated at 38°C and stained. Cells were stained using the Sigma 'Senescence Cells Histochemical Staining Kit' and incubated at 37°C over night. Blue staining, indicative of SA- $\beta$ -galactosidase activity, was visible in EcoR Clone 7 cells grown at the non-permissive temperature (Figure 4.5 A). Apart from a few cells, controls, EcoR Clone 7 cells grown at the permissive temperature and 226L U19/13 cells grown at the non-permissive temperature, did not stain for SA- $\beta$ -galactosidase (Figure 4.5 B and C). This indicates that EcoR Clone 7 cells undergo senescence arrest at the non-permissive temperature.



**Figure 4.5: Arrested EcoR Clone 7 cells stain for SA-β- galactosidase**

EcoR Clone 7 cells were grown at 34°C and 38°C for 2 weeks, and following this incubation period were stained for SA-β-gal. To control for temperature shift, 226L U19/13 cells, which express a non-thermolabile LT antigen and therefore do not arrest at 38°C, were also incubated at 38°C and stained. (A) Blue staining, indicative of SA-β-gal activity, was clearly visible in EcoR Clone 7 cells grown at the non-permissive temperature. (B) In the control EcoR Clone 7 cells grown at the 34°C and (C) 226L U19/13 cells grown at 38°C, SA-β-gal staining was not detected. This indicates that EcoR Clone 7 undergo senescence arrest at the non-permissive temperature.

#### 4.4 Discussion

Once it had been shown that 226L 8/13 cells could be used as a model to study cellular senescence, the murine ecotropic receptor was introduced into the cells. This enabled infection of the human cells with murine ecotropic viruses, and it had two advantages; firstly, it greatly increased safety, as ecotropic viruses, unlike amphotropic viruses, cannot infect human cells. Secondly, the infection efficiency of ecotropic viruses is greater than that of amphotropic viruses. 226L 8/13 cells were infected with constructs carrying the ecotropic receptor, and single cell clones were picked. This was done as for the shRNA screen, a stringent model was required. A suitable clone for the large-scale shRNA screen would need to be highly infectable with ecotropic viruses, and be highly conditional, i.e. grow at the permissive temperature but arrest at the non-permissive temperature, to ensure low background levels in the screen. Furthermore, such a clone would need to readily overcome the conditional arrest by abrogation of the p53 pathway, and also by expression of E1A and E2F-DB.

EcoR Clone 7 showed the above characteristics, and was therefore a good candidate for the screen. Once EcoR Clone 7 had been selected on the basis of the complementation assay (Figure 4.1), other characteristics such as expression of epithelial, luminal epithelial and myoepithelial markers, growth characteristics and expression of SA  $\beta$ -gal at the non-permissive temperature were tested. It was important that the Clone selected for the screen reflected the characteristics of the starting cell population.

226L 8/13 had been shown to express the epithelial markers  $\beta$ -catenin and E-cadherin, and it was demonstrated that EcoR Clone 7 cells also expressed these markers (Figure 4.2). Like in 226L 8/13 cells, in EcoR Clone 7 cells  $\beta$ -catenin was observed mainly on the membrane at cell-to-cell boundaries. E-cadherin staining was observed in the cytoplasmic membrane of EcoR Clone 7 cells, like in 226L 8/13 cells. Furthermore, as in the 226L 8/13 cells, some punctate staining was observed with the E-cadherin staining in EcoR Clone 7 cells.

Like the 226L 8/13 cells, EcoR Clone 7 cells were positive for the luminal marker cytokeratin 18 and the basal markers cytokeratin 14 and  $\beta$ -4 integrin, and they were negative for the luminal marker cytokeratin 19 (Figure 4.3). In EcoR Clone 7 cells, as

observed in 226L 8/13 cells, the intermediate filaments were visible in cytokeratin 14 and 18 staining and both had their typical appearance, and  $\beta$ -4 integrin staining also had its typical appearance.

It was demonstrated that like 226L 8/13 cells, EcoR Clone 7 cells grow at 34°C, at which temperature the temperature-sensitive LT is active, and that they cease to grow at 38°C, where the ts LT is inactive (Figure 4.4). EcoR Clone 7 cells were stained for senescence associated  $\beta$ -galactosidase, and like 226L 8/13 cells, EcoR Clone 7 cells incubated at the non-permissive temperature for two weeks were positive for this marker, whereas EcoR Clone 7 cells grown at the permissive temperature were negative (Figure 4.5). This is consistent with the growth arrest that EcoR Clone 7 cells enter at 38°C being senescence arrest.

The advantages of using a single cell clone for the screen were that this clone could be selected for stable expression of the ecotropic receptor and therefore infectivity with ecotropic viruses, as well as stringent conditionality, i.e. growth at 34°C and arrest at 38°C, and abrogation of growth arrest by inhibition of the p53 pathway and expression of E1A and E2F-DB; EcoR Clone 7 did fulfil these characteristics. The disadvantage of using a clone was that it might not truly be representative of the mixed population. By single cell cloning, a clone which does not reflect characteristics of the starting cell population, such as expression of epithelial markers, could have been selected. It was important to show that EcoR Clone 7 cells reflected the mixed cell population to select a clone for the screen that was representative of epithelial cells. But the characterisation of EcoR Clone 7 could have been done in more detail, as possible genetic mutations or epigenetic changes, such as chromosome duplications or loss of certain genes, were not investigated. Such mutations could have an effect in the complementation assay, for instance they could cause EcoR Clone 7 cells to overcome the growth arrest by knock-down of genes which would have no effect in the mixed population. Genetic alterations are not unlikely to occur, as SV40 LT antigen can cause numerical or structural chromosome aberrations, driving karyotypic instability (Ray et al., 1990). The EcoR Clone 7 cells could be karyotypically and genetically characterised to rule out such genetic alterations.



Mike O'Hare and Parmjit Jat also produced a cell line similar to 226L 8/13, in which a non-Bub1-binding ts LT antigen was used in combination with hTERT to immortalise luminal epithelial cells, and these cells are karyotypically stable. Initially, these cells were meant to be used for the screen, but unfortunately they did not arrest at the non-permissive temperature.

## **5 Results – pSM2c primary and secondary shRNAmir screen**

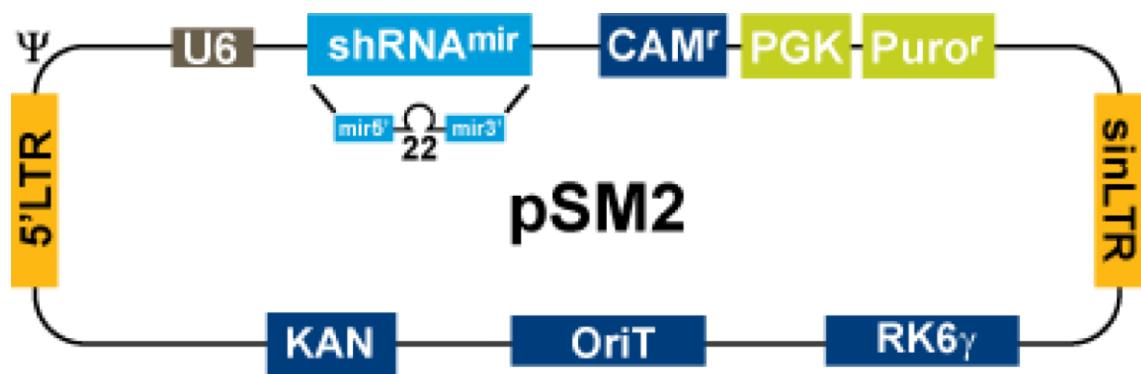
Short hairpin RNA mediated screens in mammalian cells are powerful tools for discovering the basis of loss-of-function phenotypes and have been used in various cell types to discover genes involved in various cellular processes such as embryonic stem cell identity (Ding et al., 2009) and aggregate formation in mutant Huntington in *Drosophila* (Zhang et al., 2010). The conditionally immortalised EcoR Clone 7 cells, described in the previous chapter, provide a suitable system for analysis by shRNA screening to identify genes involved in senescence. Because: (1) the EcoR Clone 7 cells exhibited a very stringent conditional growth phenotype with only low levels of background growth at the non-permissive temperature (see Figure 4.1 F and 4.4 B); (2) since inactivation of the p53 and the pRb pathways bypasses senescence in these cells, these cells have the potential of identifying targets in both of these pathways (see Figure 4.1 A, B, C and D). (3) it would be performed in a clinically highly relevant cell type, breast epithelial cells.

### **5.1 Primary screen**

#### **5.1.1 The pSM2c library**

The screen was performed using version 3 of the Cold Spring Harbor Laboratories' pShagMagic2c (pSM2c) library (Open Biosystems; gift from A. Ashworth). We received the library in 100 pools of DNA, with approximately 200 constructs per pool. The library consists of 15,148 constructs to target 9,392 genes in total, each represented by 1, 2 or 3 shRNAmir constructs (each covering a unique region of the target gene). Only 9,392 genes out of the total number of unique human genes estimated to be 22,500 (International Human Genome Sequencing Consortium, 2004) are represented in this library. However, this library is still a good tool to indentify new targets involved in senescence as these 9392 genes were enriched for cancer associated genes which makes them very relevant to studying cell cycle disruption. The library is based on the pSM2c vector (Figure 5.2), which carries the shRNA sequences under control of the U6 promoter (RNA polymerase III promoter), and conveys puromycin resistance under the control of the phosphoglycerate kinase eukaryotic promoter. The shRNAs have been

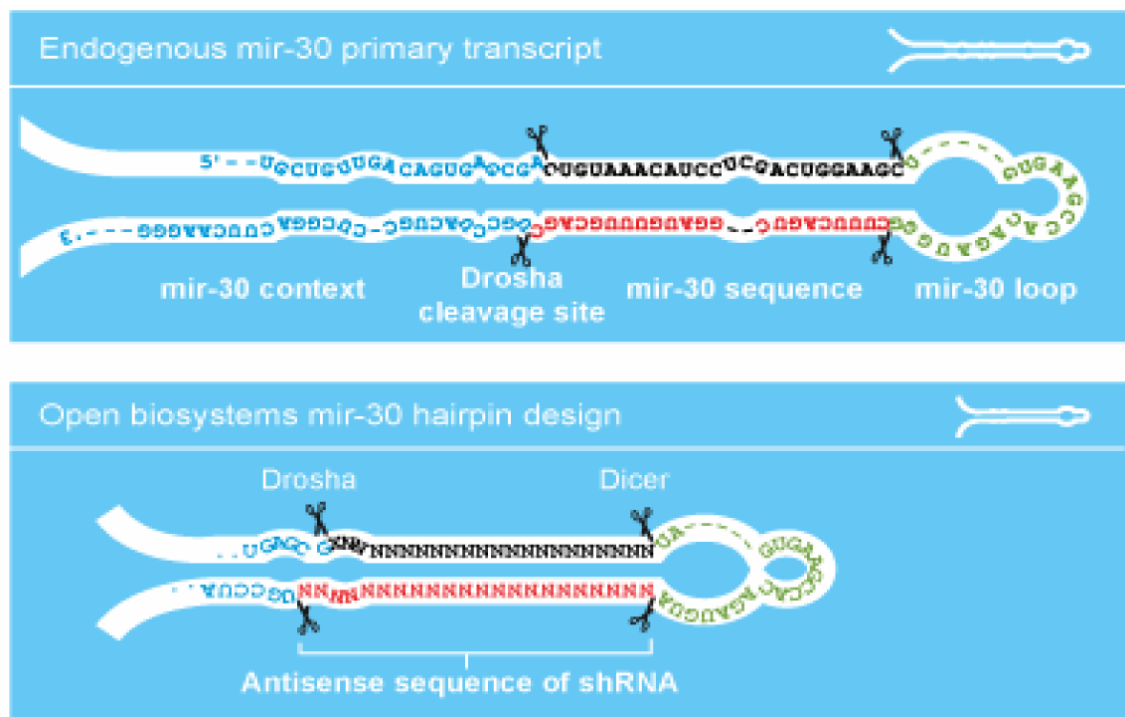
designed to mimic a natural microRNA primary transcript and are based on mir-30 by replacing the mature microRNA sequence in mir-30 with gene specific duplexes (Figure 5.3). This is meant to increase knock-down efficiency, as adding the mir-30 loop and context sequences leads to endogenous processing by Drosha which increases subsequent Dicer recognition and specificity, which in turn promotes loading of the processed siRNA onto the RISC complex. Adding a Drosha processing site to the hairpin construct has been shown to greatly increase knockdown efficiency (Boden et al., 2004). The hairpin stem consists of 22 nt of dsRNA and a 19 nt loop from the human miR30. By adding the miR30 loop and 125 nt of miR30 flanking sequence on either side of the hairpin, Drosha and Dicer processing of the expressed hairpins is increased greater than 10-fold compared to conventional shRNA designs without these features (Silva et al., 2005). Before the siRNA is loaded onto RISC, the two strands of the duplex are separated resulting in departure of the passenger strand (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005). Selection of the RNA strand to be incorporated is governed by the thermodynamic profile of the siRNA duplex termini. The siRNA strand whose 5' end is more weakly bound to the complementary strand more readily incorporates into RISC (Khvorova et al., 2003b; Schwarz et al., 2003; Tomari et al., 2004). The hairpins in this library are designed to destabilize the 5' end of the antisense strand for strand specific incorporation into RISC.



Vector Element	Utility
U6 Promoter	RNA generated with four uridine overhangs at each 3' end
PGK	phosphoglycerate kinase eukaryotic promoter
puroR	Puromycin resistance for mammalian selection
5'LTR	5' long terminal repeat
SIN-LTR	3' self inactivating long terminal repeat
RK6g	Conditional origin of replication. Requires the expression of pir1 gene within the bacterial host to propagate
Kanr/CAMr	Bacterial selectable marker

**Figure 5.2: pSM2 vector design**

The pSM2 vector carries the shRNA sequences under control of the U6 promoter (RNA polymerase III promoter), and conveys puromycin resistance under the control of the phosphoglycerate kinase eukaryotic promoter ("pSM2 Retroviral shRNAmir" - Thermo Scientific Open Biosystems: <http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral/>).



**Figure 5.3: miR-30 adapted shRNAmir transcript design**

The shRNAs have been designed to mimic a natural microRNA primary transcript and are based on mir-30 by replacing the mature microRNA sequence in mir-30 with gene specific duplexes ("shRNAmir design" - Thermo Scientific Open Biosystems: <http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral/shRNAmirDesign/>).

### 5.1.2 Sensitivity

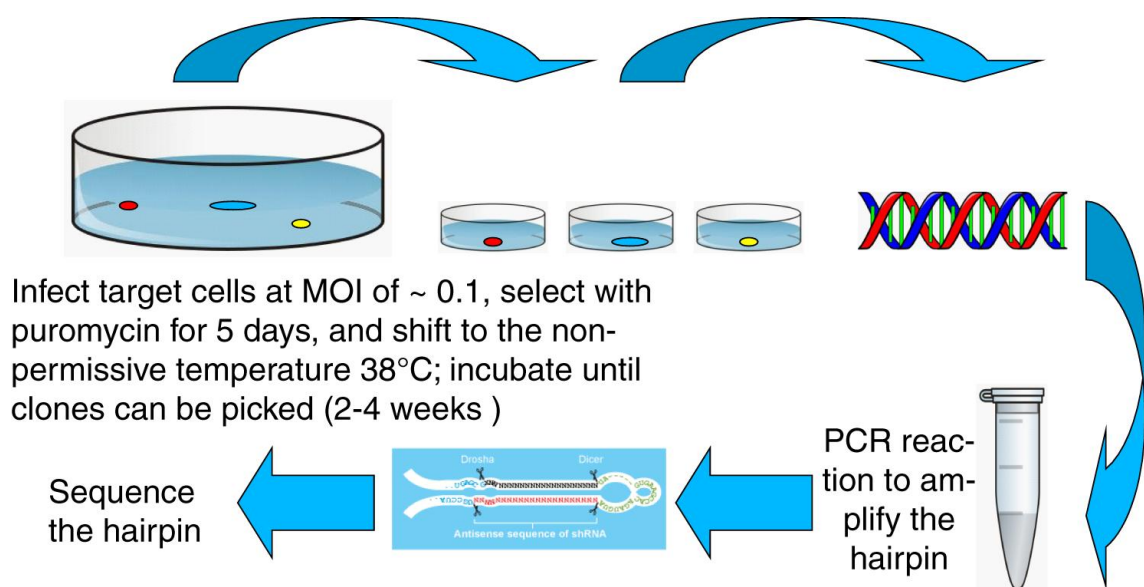
It was important to minimise the background levels of false-positive hits without losing true positive hits. The optimal conditions for performing an RNAi screen in the EcoR Clone 7 system had been determined by the development of the EcoR Clone 7 complementation assay (see Figure 4.1), with an optimal seeding density of approximately 1,050 cells per cm<sup>2</sup> (80,000/T75 or 10,000/well in 6-well plates) for growth at the non-permissive temperature, 38°C. But for the screen the protocol was modified slightly; first, it was established that puromycin selection could already be added 24 hours post-infection. Second, cells were infected at an MOI of 0.1 to achieve single inserts. This was to enable identification of inserts that led to cells overcoming the conditional arrest directly by PCR amplification and sequencing of the insert. Third, ideally cells should not be reseeded before being shifted to the non-permissive temperature. In the complementation assay it had been established that the ideal number of cells to be reseeded was 10,000 per well in 6-well plates, but that reseeding approximately 5,000 to 20,000 cells was within the limits of the assay (Figure 4.1). This corresponds to approximately 40,000 to 156,000 cells in T75 flasks, or 80,000 to 312,000 cells in T150 flasks. Therefore EcoR Clone 7 cells were seeded at 500,000 cells in T75 flasks or at 1,000,000 cells in T150 flasks, and infected at an MOI of 0.1, leaving approximately 50,000 or 100,000 puromycin-resistant cells respectively. It was found that following this strategy cells did not need to be reseeded before they were shifted to the non-permissive temperature as clones were small and well-separated following drug-selection. Not reseeding cells is advantageous as by reseeding cells from one growing colony might produce several growing colonies once reseeded. If cells are not reseeded and an insert is identified multiple times this suggest that this insert is a true positive, whereas if cells are reseeded clones carrying the same insert might all stem from the same infected cell.

The library consisted of 100 pools, each containing approximately 200 constructs, and before the screen was carried out, it was investigated if the complementation assay was sensitive enough to detect constructs which were able to abrogate the growth arrest in the pools when following the protocol designed for the screen. To test for sensitivity, a

1 in 200 mix of pRetroSuper-p21 positive control DNA in pRetroSuper-LaminA/C negative control DNA was packaged and used to infect 1,000,000 EcoR Clone 7 cells at an MOI of 0.1. Following incubation at the non-permissive temperature for 2 weeks, multiple colonies were observed (data not shown), demonstrating that the complementation assay was sensitive enough to detect constructs which were able to abrogate the growth arrest.

### **5.1.3 Infection of EcoR Clone 7 cells and growth conditions**

EcoR Clone 7 cells were frozen in bulk at passage 12/13. For each pool, cells were infected at passage 14; this was done to reduce the chance of background caused by mutations that cells could acquire due to extensive passageing and also to ensure that all pools were screened under the same conditions. As explained in Chapter 2.8, 919 infectious events per pool were required to achieve a 99% confidence interval to cover each construct in the pool. For pools 1-20 EcoR Clone 7 cells were seeded in duplicate at 500,000 cells in T75 flasks the day before transduction, and infected over night at an MOI of approximately 0.1 in the presence of 8 µg/ml polybrene and 15 ml complete medium. For pools 21-100 cells were seeded at 1,000,000 cells in T150 flasks and were infected over night at an MOI of approximately 0.1 in the presence of 8 µg/ml polybrene and 25 ml of complete medium. This equals approximately 100,000 infectious events for each pool. Following puromycin selection, most pools were shifted to 38°C without being reseeded. Pools 3, 5, 11, and 20 were reseeded at 180,000 cells per T175 or approximately 80,000 cells in T75 flasks as puromycin-resistant clones were not well separated following drug selection and very confluent cultures of cells could have produced higher levels of background. pRetroSuper-LaminA/C transduced cells were used as the negative control, and pRetroSuper-p21 transduced cells were used as the positive control. The pSM2c p53 shRNA construct V2HS\_93615, which is present in pool 82 of the library, was not available, and therefore could not be used as the positive control. Cells were fed twice per week, and after two to three weeks of incubation at the non-permissive temperature clones that overcame the conditional arrest could be detected. These were ring-cloned and expanded for DNA extraction. PCR reactions were set up to amplify the insert, which was then sequenced. Identified hairpins were tested in a secondary screen. Figure 5.1 illustrates the strategy of the screen.



**Figure 5.1: shRNA screening strategy**

Illustration of the screening protocol. EcoR Clone 7 passage 14 cells were infected at an MOI of 0.1. Following infection, selection with 2µg/ml puromycin was applied for 5 days, after which cells were shifted to the non-permissive temperature, 38°C. Cultures were grown at the non-permissive temperature until growing colonies could be ring-cloned, which were then expanded for DNA extraction. PCR reactions were set up to amplify the insert, which was then sequenced (figure adapted from Open Biosystems; "shRNAmir design" - Thermo Scientific Open Biosystems: <http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral/shRNAmirDesign/>).



#### **5.1.4 Retroviral production and titrations**

10 µg of DNA of each pool were packaged using Φ ecotropic retroviral packaging cells. 11 ml of supernatant were harvested 48 hours and 72 hours post-transfection. Of each harvest, one 1 ml and one 10 ml aliquot were frozen and stored at -70°C. The 1 ml aliquots were used to determine the titer of the supernatant. The 48h and 72h harvests were mixed. To determine the titer, EcoR Clone 7 cells were seeded at 64,000 cells/well in 6 well plates and infected over night with 1 µl, 10 µl, 20 µl, 50 µl, 100 µl, and 500 µl of the virus in the presence of 8 µg/ml polybrene and 2ml of complete medium. 24 hours post-transduction, 2µg/ml puromycin was added to the culture medium; following 5 days of selection, cells were grown for 1-2 weeks without puromycin, stained with methylene blue, and clones of growing cells were counted to determine the titer of each pool. Table 5.1 shows the titer of each pool and the amount of virus required to infect 1,000,000 cells at an MOI of 0.1, i.e. the amount of virus required for 100,000 infectious events.

Pool	Titre (TU/ml)	Virus (ml) required to achieve 100,000 infectious events	Pool	Titre (TU/ml)	Virus (ml) required to achieve 100,000 infectious events
1	5333	19	51	5667	18
2	7000	14	52	6000	17
3	6333	16	53	8000	13
4	5333	19	54	7000	14
5	9000	11	55	6000	17
6	8333	12	56	6667	15
7	5333	19	57	9333	11
8	5667	18	58	5000	20
9	7000	14	59	3000	33
10	5333	19	60	6333	16
11	6000	17	61	5667	18
12	7333	14	62	7000	14
13	10667	9	63	6333	16
14	6333	16	64	7333	14
15	5333	19	65	5333	19
16	8333	12	66	7333	14
17	5667	18	67	6000	17
18	8667	12	68	7333	14
19	8000	13	69	8000	13
20	3667	27	70	7000	14
21	4000	25	71	11667	9
22	8000	13	72	7333	14
23	6667	15	73	5000	20
24	7000	14	74	6333	16
25	7000	14	75	8000	13
26	8000	13	76	4333	23
27	5333	19	77	8333	12
28	7333	14	78	3000	33
29	6667	15	79	8000	13
30	6000	17	80	8333	12
31	6333	16	81	8000	13
32	5333	19	82	5333	19
33	7000	14	83	8333	12
34	7000	14	84	5000	20
35	5667	18	85	7333	14
36	8667	12	86	6333	16
37	10667	9	87	6667	15
38	6333	16	88	6000	17
39	7333	14	89	10667	9
40	10333	10	90	7333	14
41	7667	13	91	6333	16
42	4333	23	92	7000	14
43	7000	14	93	7000	14
44	7667	13	94	7000	14
45	12000	8	95	8000	13
46	5333	19	96	13333	8
47	4333	23	97	5667	18
48	8667	12	98	8333	12
49	9333	11	99	7333	14
50	6667	15	100	6333	16

**Table 5.1: pSM2c pool titers**

The titer and the amount of virus required to infect 1,000,000 cells at an MOI of 0.1 (i.e. the amount of virus required for 100,000 infectious events) was determined for each pool.

### **5.1.5 Identification and isolation of shRNA inserts in clones of growing cells which overcame the conditional arrest**

Clones which were growing at the non-permissive temperature were grown to sufficient size and then ring-cloned and seeded in 24 well plates; if several clones were detected in one flask all of them were picked. At the non-permissive temperature, clones were grown to confluence in 24 well plates, and then split into 6 well plates where they were grown to confluence before cells were harvested and genomic DNA extracted. Of the clones picked, some arrested either at the 24 well plate- or the 6 well plate-stage, and therefore DNA was not extracted from such clones. A total of 110 clones were picked from the screen, and 101 of these continued to grow. Table 5.2 shows the number of clones picked per pool, and the number of clones that continued to grow.

200ng genomic DNA was used in 50 µl PCR reactions that contained the pSM2 specific primers pSM2longForward (GCAGCACATATACTAGTCGACTAGGGATAACAGG) and pSM2longReverse (CGAAGTGATCTTCCGTCACAGGTTTTATTC). 5 µl of each PCR reaction was then resolved alongside 5 µl 1kb+ DNA ladder on a 1.4% agarose gel to check for the generation of the 438 bp PCR products. For some of the DNA samples, PCR reactions failed; each of these reactions was set up again, but if they failed again they were not repeated. This could have been due to loss of the insert, or a mutation which led to primers not binding or loss of the sites corresponding to the primers. It is also possible that for these DNA samples the PCR amplification was not very efficient, and in that case nested PCR could have been used to amplify the inserts; but due to the high number of shRNAs identified in the screen such inserts were not pursued. PCR products were purified using the micro-CLEAN system, and sequencing was carried out by the genetics group within the MRC Prion unit. The pSM2shortForward primer was used for the sequencing reaction. Three of the sequencing reactions were unsuccessful. To identify inserts from total sequenced area, the hairpin framing sequence [the miR-30 context and miR-30 loop; common to all the constructs; (Figure 5.3)] was located. The sequence framed by this, the mature siRNA sequence, was then looked for in the pSM2c data base, which contains all hairpin sequences and available information such as which genes the hairpins were designed to target. When the screen was first started,

siRNA sequences were BLASTED against the NCBI human genome data to identify the target genes.

From the 101 clones from which DNA was extracted, 87 yielded 438 bp inserts, and 84 of these were successfully sequenced (Table 5.2). These led to the identification of 63 individual shRNA constructs. Table 5.3 lists candidate genes and the shRNAs detected in the screen.

Pool	Number of clones picked per	Number of clones that continued to grow	Number of shRNA inserts identified	500bp PCR products
1	0	0	0	0
2	0	0	0	0
3	18	18	17 (one 5x and one 7x)	18
4	1	1	1	1
5	9	9	7 (same shRNA 7 times)	7
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
11	2	2	2 (same shRNA twice)	2
12	1	1	1	1
13	1	0	0	0
14	0	0	0	0
15	4	2	1	1
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	3	3	3 (same shRNA 3 times)	3
21	0	0	0	0
22	0	0	0	0
23	0	0	0	0
24	0	0	0	0
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0
31	0	0	0	0
32	0	0	0	0
33	0	0	0	0
34	0	0	0	0
35	0	0	0	0
36	1	1	1	1
37	0	0	0	0
38	1	1	1	1
39	0	0	0	0
40	0	0	0	0
41	0	0	0	0
42	0	0	0	0
43	0	0	0	0
44	1	1	1	1
45	0	0	0	0
46	0	0	0	0
47	0	0	0	0
48	0	0	0	0
49	1	1	0	0
50	0	0	0	0

Pool	Number of clones picked per	Number of clones that continued to grow	Number of shRNA inserts identified	500bp PCR products
51	0	0	0	0
52	0	0	0	0
53	0	0	0	0
54	1	1	0	0
55	0	0	0	0
56	5	5	5 (one shRNA twice)	5
57	3	3	3	3
58	1	1	1	1
59	2	2	1	1
60	0	0	0	0
61	0	0	0	0
62	1	1	1	1
63	0	0	0	0
64	0	0	0	0
65	2	1	1	1
66	0	0	0	0
67	1	1	1	1
68	0	0	0	0
69	0	0	0	0
70	0	0	0	0
71	0	0	0	0
72	0	0	0	0
73	1	1	1	1
74	2	1	1	1
75	0	0	0	0
76	0	0	0	0
77	0	0	0	0
78	0	0	0	0
79	0	0	0	0
80	1	1	1	1
81	5	5	5 (one shRNA twice)	5
82	5	4	4	4
83	3	3	2	2
84	3	3	2	2
85	5	2	1	1
86	1	1	1	1
87	1	1	1	1
88	2	2	0	1
89	1	1	0	0
90	2	2	2	2
91	6	6	5	5
92	0	0	0	0
93	2	2	1	2
94	1	1	1	1
95	1	1	1	1
96	2	2	2	2
97	2	2	2	2
98	3	3	1	1
99	2	2	2	2
100	0	0	0	0
<b>Total</b>	<b>110</b>	<b>101</b>	<b>84 (63 individual shRNAs)</b>	<b>87</b>

**Table 5.2: pSM2c primary screen summary**

A total of 110 clones were picked from the screen, and 101 of these continued to grow and DNA was extracted. 87 yielded 438 bp inserts, and 84 of these were successfully sequenced. These led to the identification of 63 individual shRNA constructs.

Pool	Reseeded	shRNA identified
3	yes	TOAK1: V2HS_59892 GLS2: V2HS_71051 (7x) NDC80: V2HS_65600 LOC219958: V2HS_66916 CLEC5A: V2HS_71374 (5x) LOC344307: V2HS_55251 PRO0397: V2HS_58944
4	no	FLJ3600: V2HS_57466
5	yes	CCCTCAGCGTCTCCAATTACAT (7x)
11	yes	OXGR1: V2HS_58850 (2x)
12	no	LOC348891: V2HS_65035
15	no	LOC344635: V2HS_55092
20	yes	DSC2: V2HS_62007 (3x)
36	no	LOC351318: V2HS_51318
38	no	LOC219321: V2HS_53095
44	no	SLC12A6: V2HS_49730
56	no	Rab23: V2HS_115231 (2x) LOC284651: V2HS_122068 TKTL2: V2HS_117914 GOT1: V2HS_114214
57	no	FMR1: V2HS_113928 LOC350158: V2HS_125697 ZBP1: V2HS_116790
58	no	LOC346878: V2HS_125075
59	no	LOC284034: V2HS_121535
62	no	LOC341206: V2HS_122567
65	no	CASC2: V2HS_118633
67	no	LOC221710: V2HS_102282
73	no	LOC351169: V2HS_110617
74	no	LOC350575: V2HS_109881
80	no	LOC349906: V2HS_108927
81	no	CNOT6: V2HS_96585 (2x) CSRP2BP: V2HS_100126 LOC346665: V2HS_107698 CD53: V2HS_93671
82	no	LOC645231: V2HS_110270 LOC346696: V2HS_107848 LOC350585: V2HS_109826 LOC729686: V2HS_101013 (not available as pGIPZ, instead V2LHS_64780)
83	no	LOC339526: V2HS_104148 LOC151647: V2HS_101815
84	no	LOC349975: V2HS_109096 MGC5566: V2HS_98782 (not available as pGIPZ, instead V2LHS_98781, V2LHS_98784)
85	no	LOC149301: V2HS_101511
86	no	COQ6: V2HS_134428
87	no	KCNMA1: V2HS_133674
90	no	FLJ21075: V2HS_136662 LOC222119: V2HS_141264
91	no	RPL26: V2HS_131626 KPNA3: V2HS_133768 CHCHD8: V2HS_135019 coronin 7: V2HS_136224 V2HS_136793 (could not determine what it targets; not available as pGIPZ; not tested in secondary screen)
93	no	LOC340536: V2HS_143839
94	no	GRM6: V2HS_130905
95	no	IL12RB2: V2HS_132321
96	no	IRF-1: V2HS_133394 GRB2: V2HS_137365
97	no	LOC352177: V2HS_148642 LOC352208 : V2HS_148744
98	no	KPNA1: V2HS_133758
99	no	ZNF454: V2HS_141497 LOC346321: V2HS_145373

**Table 5.3 pSM2c primary screen: shRNAs identified**

This table lists candidate genes and the shRNAs detected in the screen.

## 5.2 Secondary screen

A total of 63 individual shRNAs had been identified in the primary screen, and these candidates were all tested in a secondary screen. For validation in a secondary screen, hairpins that were successfully identified were then obtained from the Open Biosystems lentiviral pGIPZ library rather than the pSM2c library, as UCL had joined the Open Biosystems shRNAir consortium and we therefore had free access to the pGIPZ library. Generally, the shRNAs in the pGIPZ library are the same as in the pSM2c library, only in a different vector backbone. If shRNAs were replaced by others in the pGIPZ library, this is due to the newer shRNAs silencing better. In most cases the same hairpin as detected in the screen was obtained from the pGIPZ library, and where the same construct was not available all other available hairpins for the target gene were tested in a secondary screen. At the start of the screen, isolated sequences were BLASTED against the NCBI human genome database to identify the target genes. In these cases all available hairpins targeting the identified genes were tested in a secondary screen, not only the one isolated in the primary screen. Furthermore, sometimes BLASTING the sequences led to identification of several potential target genes, in which case all available shRNAs against all of these were tested. For a sequence identified in pool 5 an exact match could not be found, therefore several hairpins targeting three genes similar to the sequence identified were tested in a secondary screen. As for the primary screen, EcoR Clone 7 cells frozen at passage 12/13 were used for the validation screen. The complementation assay was used as described in section 3.5.1, with three modifications: Puromycin selection was started 24 hours instead of 48 hours post-infection at 6µg/ml instead of 2µg/ml. Also, cells were infected at a high MOI for the secondary screen (5 ml of virus per 80,000 cells), as a higher MOI was shown to produce better knock-down and rescue with the positive control shRNAs targeting p21, and better rescue with positive control shRNAs targeting p53 (Figure 6.6 and 6.7). pGIPZ non-silencing control (scrambled) was used as a negative control.

In total, 95 shRNAs were tested in the secondary screen, targeting 67 candidate genes. Out of these, 5 led to EcoR Clone 7 cells overcoming the conditional arrest in the secondary screen. Table 5.4 summarizes candidate genes, the specific shRNAs isolated in the screen, and shRNAs tested for each candidate gene.



Pool	Gene name	shRNAs identified in the primary screen	shRNAs tested in the secondary screen
3	TOAK1	V2HS_59892	V2LHS_59892, V2LHS_202490, V2LHS_202996, V2LHS_261946, V2LHS_201624
	GLS2	V2HS_71051 identified in 7 clones	V2LHS_71051, V2LHS_71048, V2LHS_71049, V2LHS_71050
	NDC80	V2HS_65600	V2LHS_65600, V2LHS_65601, V2LHS_263291, V2LHS_247761
	LOC219958	V2HS_66916	V2LHS_66916
	MEGF10	(V2HS_66916)	V2LHS_138972, V2LHS_138974, V2LHS_138969
	CLEC5A	V2HS_71374 identified in 5 clones	V2LHS_71374, V2LHS_71376, V2LHS_71372, V2LHS_71373
	LOC344307	V2HS_55251	V2LHS_55251
	PRO0397	V2HS_58944	V2LHS_58944
	FLJ3600	V2HS_57466	V2LHS_57466
	LOC100131001	(V2HS_57466)	V2LHS_57466, V2LHS_57463
4	NFASC	(V2HS_57466)	V2LHS_205442
	CACNA1H	CCCTCAGCGTCTCCAATTACAT identified in 7 clones	V2LHS_72193, V2LHS_72189
	CACNA1G	CCCTCAGCGTCTCCAATTACAT	V2LHS_20824, V2LHS_20823, V2LHS_20822, V2LHS_20825, V2LHS_20821
5	KIAA0240	CCCTCAGCGTCTCCAATTACAT	V2LHS_233585
	OXGR1	V2HS_58850 identified in 2 clones	V2LHS_58850, V2LHS_58922, V2LHS_58923
11	LOC348891	V2HS_65035	V2LHS_65035
12	LOC344635	V2HS_55092	V2LHS_55092
15	DSC2	V2HS_62007 identified in 3 clones	V2LHS_62007, V2LHS_62042, V2LHS_62044, V2LHS_62043 and V2LHS_62040
20	LOC351318	V2HS_51318	V2LHS_51318
36	LOC219321	V2HS_53095	V2LHS_53095
38	SLC12A6	V2HS_49730	V2LHS_49730
44	Rab23	V2HS_115231 identified in 2 clones	V2LHS_115231
56	LOC284651	V2HS_122068	V2LHS_122068
	TKTL2	V2HS_117914	V2LHS_117914
	GOT1	V2HS_114214	V2LHS_114214
	FMR1	V2HS_113928	V2LHS_113928
57	LOC350158	V2HS_125697	V2LHS_125697
	ZBP1	V2HS_116790	V2LHS_116790
58	LOC346878	V2HS_125075	V2LHS_125075
59	LOC284034	V2HS_121535	V2LHS_121535
62	LOC341206	V2HS_122567	V2LHS_122567
65	CASC2	V2HS_118633	V2LHS_118633
67	LOC221710	V2HS_102282	V2LHS_102282
73	LOC351169	V2HS_110617	V2LHS_110617
74	LOC350575	V2HS_109881	V2LHS_109881
80	LOC349906	V2HS_108927	V2LHS_108927
81	CNOT6	V2HS_96585 identified in 2 clones	V2LHS_96585
	CSRP2BP	V2HS_100126	V2LHS_100126
82	LOC346665	V2HS_107698	V2LHS_107698
	CD53	V2HS_93671	V2LHS_93671
	LOC645231	V2HS_110270	V2LHS_110270
	LOC346696	V2HS_107848	V2LHS_107848
83	LOC350585	V2HS_109826	V2LHS_109826
	LOC729686	V2HS_101013	V2LHS_64780 (V2LHS_101013 not available in pGIPZ)
	LOC339526	V2HS_104148	V2LHS_104148
	LOC151647	V2HS_101815	V2LHS_101815
84	LOC349975	V2HS_109096	V2LHS_109096
85	MGC5566	V2HS_98782	V2LHS_98781, V2LHS_98784 (V2LHS_98782 not available in pGIPZ)
	LOC149301	V2HS_101511	V2LHS_101511
86	COQ6	V2HS_134428	V2LHS_134428
87	KCNMA1	V2HS_133674	V2LHS_133674
90	FLJ21075	V2HS_136662	V2LHS_136662
	LOC222119	V2HS_141264	V2LHS_141264
91	RPL26	V2HS_131626	V2LHS_131626
	KPNA3	V2HS_133768	V2LHS_133768
	CHCHD8	V2HS_135019	V2LHS_135019
	coronin 7	V2HS_136224	V2LHS_136224
93	LOC340536	V2HS_143839	could not determine what it targets; not available as pGIPZ; not tested in secondary screen
94	GRM6	V2HS_130905	V2LHS_143839
95	IL12RB2	V2HS_132321	V2LHS_130905
96	IRF-1	V2HS_133394	V2LHS_132321
97	GRB2	V2HS_137365	V2LHS_133394
	LOC352177	V2HS_148642	V2LHS_137365
98	LOC352208	V2HS_148744	V2LHS_148642
	KPNA1	V2HS_133758	V2LHS_148744
99	ZNF454	V2HS_141497	V2LHS_133758
	LOC346321	V2HS_145373	V2LHS_141497

**Table 5.4: pSM2c secondary screen: candidate genes and shRNAs tested**

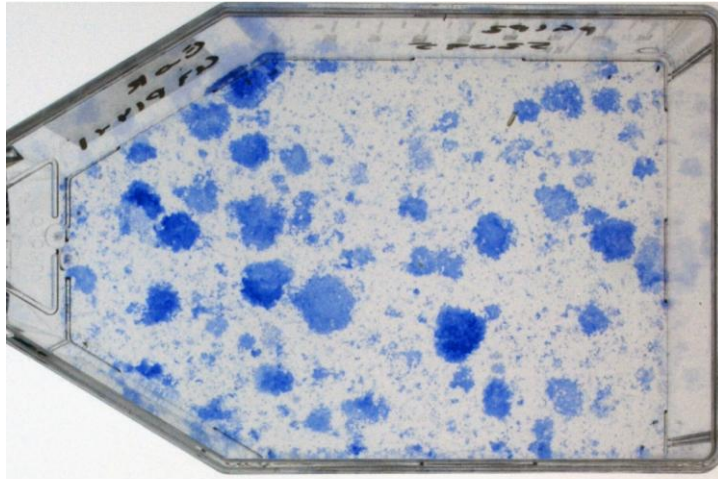
This table summarizes candidate genes, the specific shRNAs isolated in the screen, and shRNAs tested for each candidate gene. Highlighted in yellow are shRNAs validated in the secondary screen.

### **5.2.1 Candidate shRNAs validated in the secondary screen**

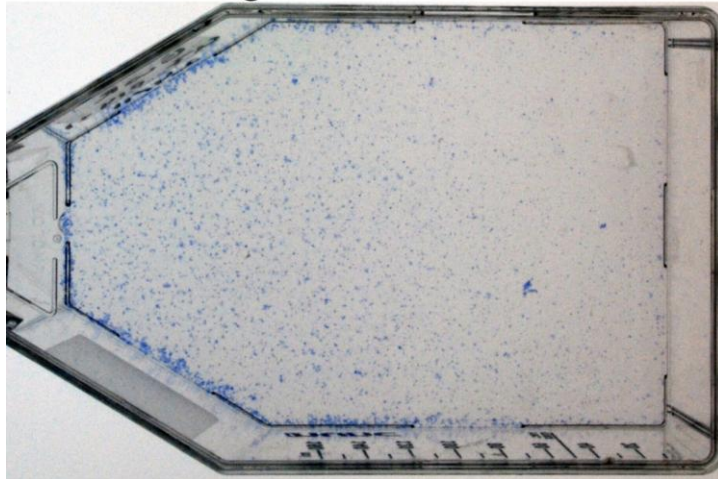
#### **V2LHS\_53095**

The shRNA construct V2HS\_53095 was isolated in pool 38, and it was only isolated once. This hairpin is designed to knock down LOC219321. The same hairpin was available from the pGIPZ library, and V2LHS\_53095 was tested in the complementation assay. Very few growing colonies were visible in cells infected with the non-silencing control virus, whereas many large colonies of growing cells were observed in cells infected with the hairpin 53095. The experiment was set up in triplicate and representative flasks are shown in Figure 5.4. This demonstrated that V2LHS\_53095 was sufficient to suppress the temperature-shift-induced proliferation arrest.

### LOC219321 shRNA 53095



### Non-silencing



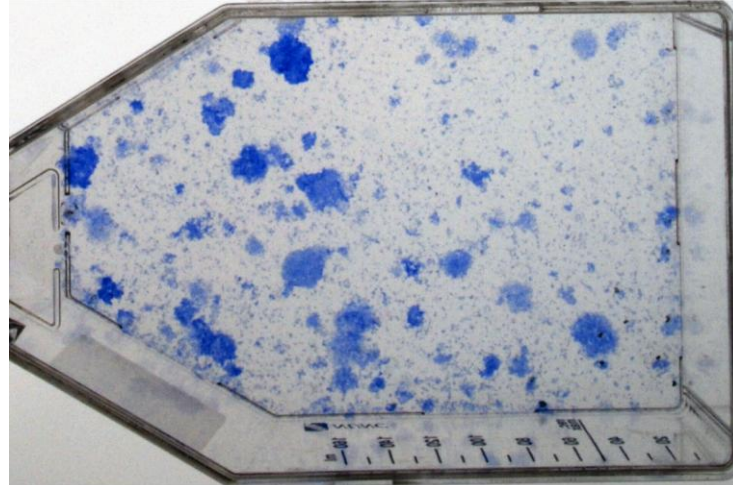
**Figure 5.4: V2LHS\_53095 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The LOC219321 shRNA construct V2LHS\_53095 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 53095, and only very low background levels were detected in cells infected with the non-silencing control virus.

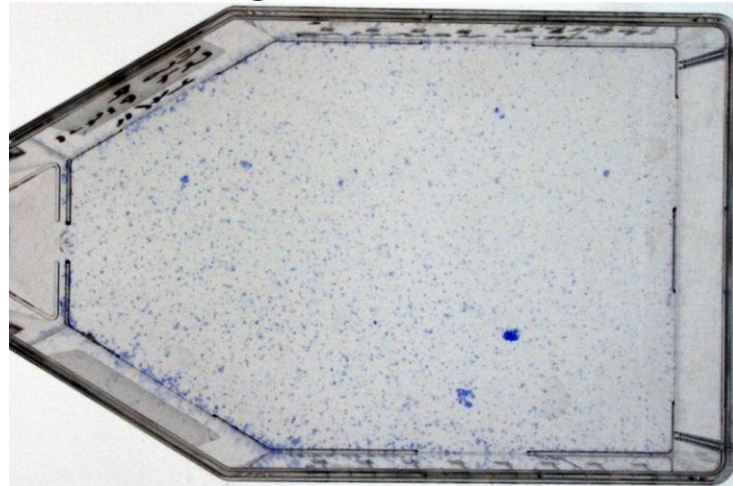
## **V2HS\_115231**

The shRNA construct V2HS\_115231 was isolated in two individually picked clones in pool 56. This shRNA silences Rab23, and the same hairpin was available from the pGIPZ library. V2LHS\_115231 was tested in the complementation assay. Following incubation at the non-permissive temperature, very low background levels were detected in non-silencing control infected cells, but many healthy looking colonies of growing cells were detected in Rab23 shRNA infected cells. The experiment was set up in triplicate and representative flasks are shown in Figure 5.5. This showed that the hairpin 115231 was sufficient to overcome the conditional growth arrest of EcoR Clone 7 cells.

### **Rab 23 shRNA 115231**



### **Non-silencing**



**Figure 5.5: V2LHS\_115231 is sufficient to abrogate the EcoR Clone 7 growth arrest**

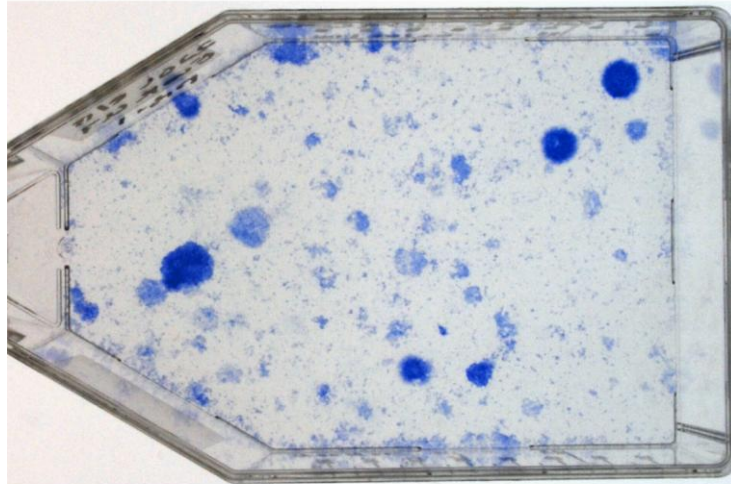
The Rab23 shRNA construct V2LHS\_115231 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 115231, and only very low background levels were detected in cells infected with the non-silencing control virus.

### **V2HS\_109096**

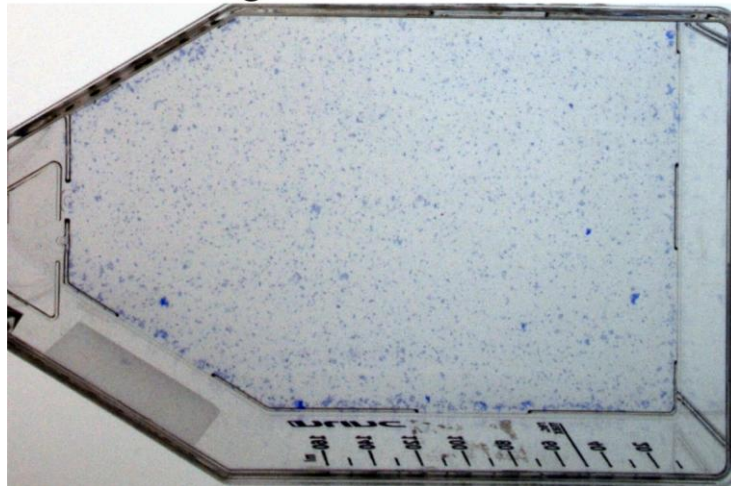
The shRNA construct V2HS\_109096 was isolated in pool 84. It is designed to knock-down LOC349975. The same hairpin was available from the pGIPZ library, and V2LHS\_109096 was tested in the complementation assay for its ability to overcome the EcoR Clone 7 growth arrest. Following 2 weeks of incubation at the non-permissive temperature, very few colonies of growing cells were detected in the non-silencing control infected cells, but multiple healthy looking colonies of growing cells were detected in cells infected with the hairpin 109096. The experiment was set up in triplicate and representative flasks are shown in Figure 5.6. This showed that the shRNA was sufficient to suppress the conditional growth arrest.



### LOC349975 shRNA 109096



### Non-silencing



**Figure 5.6: V2LHS\_109096 is sufficient to abrogate the EcoR Clone 7 growth arrest**

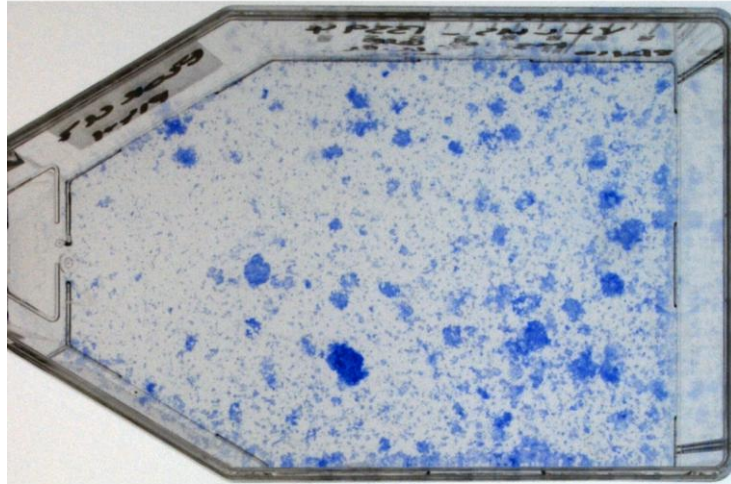
The LOC349975 shRNA construct V2LHS\_109096 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 109096, and only very low background levels were detected in cells infected with the non-silencing control virus.

## **V2HS\_133394**

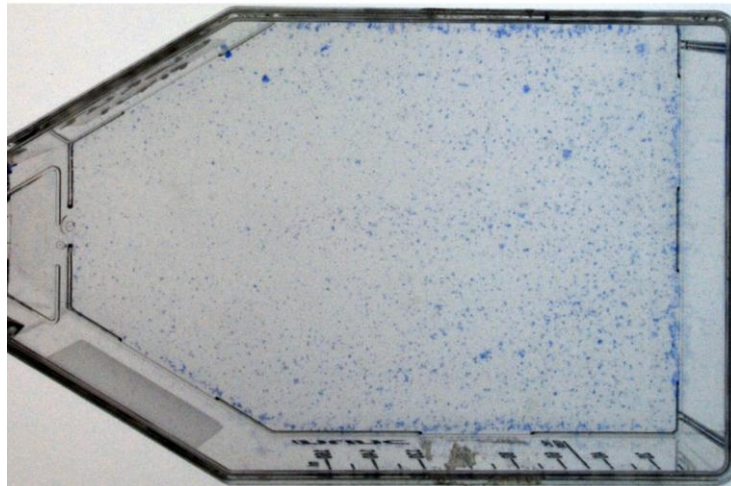
In pool 96 the IRF-1 shRNA construct V2HS\_133394 was detected in one clone. The same hairpin was obtained from the pGIPZ library, and V2LHS\_133394 was tested in the EcoR Clone 7 complementation assay. After 2 weeks of incubation at the non-permissive temperature, very low background levels were detected in the non-silencing control infected cultures, whereas many healthy looking colonies of growing cells were detected in the IRF-1 shRNA infected cells. The experiment was set up in triplicate and representative flasks are shown in Figure 5.7. This demonstrated that the shRNA 133394 was sufficient to overcome the temperature-shift-induced growth arrest of EcoR Clone 7 cells.



### IRF-1 shRNA 133394



### Non-silencing



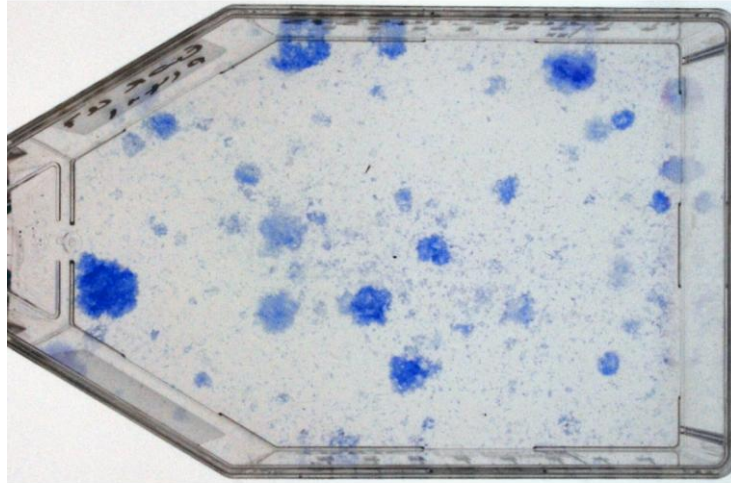
**Figure 5.7: V2LHS\_133394 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The IRF-1 shRNA construct V2LHS\_133394 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 133394, and only very low background levels were detected in cells infected with the non-silencing control virus.

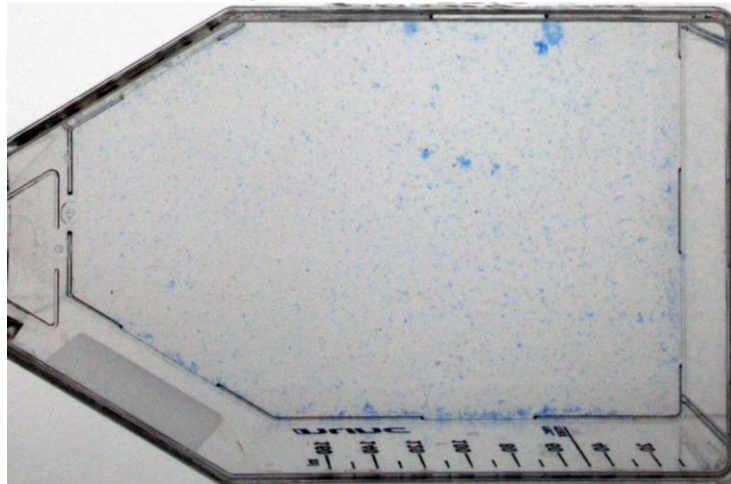
### **V2HS\_145373**

In pool 99 the shRNA construct V2HS\_145373 was isolated from one clone. This hairpin is designed to knock-down LOC346321, and the same shRNA was available from the pGIPZ library. V2LHS\_145373 was tested in the complementation assay. Following 2 weeks of incubation at the non-permissive temperature very low background levels were detected in the non-silencing control-infected cells, but multiple colonies of growing cells were detected in cultures infected with the shRNA 145373. The experiment was set up in triplicate and representative flasks are shown in Figure 5.8. This showed that the hairpin 145373 was sufficient to abrogate the conditional growth arrest of EcoR Clone 7 cells.

### LOC346321 shRNA 145373



### Non-silencing



**Figure 5.8: V2LHS\_145373 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The LOC346321 shRNA construct V2LHS\_145373 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 145373, and only very low background levels were detected in cells infected with the non-silencing control virus.

### 5.3 Discussion

A loss of function shRNAmir screen was carried out in the conditional EcoR Clone 7 cells to identify mediators of cellular senescence in human breast epithelial cells. The pSM2c library, which consists of 15,148 constructs targeting 9,392 genes in total, each represented by 1, 2 or 3 shRNAmir constructs, was screened. Only 9,392 genes out of the total number of unique human genes, estimated to be 22,500 (International Human Genome Sequencing Consortium, 2004) are represented in this library. However, this library was still a good tool to identify new targets involved in senescence as these 9392 genes were enriched for cancer associated genes which makes them very relevant to studying cell cycle disruption. The screen was performed in 100 pools of approximately 200 constructs each, and for each pool approximately 1,000,000 cells were infected at an MOI of approximately 0.1, which corresponds to 100,000 infectious events per pool. A total of 110 clones were ring-cloned from the 100 pools, and from these 110 clones 84 hairpins were identified (Table 5.2). These led to the identification of 63 individual shRNA constructs. Table 5.3 lists candidate genes and the shRNAs detected in the screen. Out of these, 5 led to EcoR Clone 7 cells overcoming the conditional arrest in a secondary screen (Table 5.4).

#### 5.3.1 Sensitivity and saturation

The effectiveness of the screen was dependent upon its sensitivity and stringency, and in this respect it was important to minimize background levels whilst still detecting all true positive hits. It was important to find a balance between allowing true positives to be detected, whilst minimizing false positives which would increase the workload. To achieve this, optimal conditions for performing the shRNAmir screen in the EcoR Clone 7 cells were determined by the development of the EcoR Clone 7 complementation assay (Figure 4.1). The frequency of the event which was being searched for needed to be considered to achieve sufficient sensitivity. The library consisted of 100 pools, each containing approximately 200 constructs. Therefore, to test for sensitivity, a 1 in 200 mix of pRetroSuper-p21 positive control in pRetroSuper-LaminA/C negative control virus was used to infect 1,000,000 cells at an MOI of 0.1. Multiple colonies were

observed, demonstrating that the complementation assay was sensitive enough to detect constructs which were able to abrogate the growth arrest.

Furthermore, to be 99% confident that the entire library was screened, i.e. the screen is saturated, the library needed to be searched through approximately 4 times its size, as determined by the following equation:  $\{\ln(1-0.99)/\ln(1-1/(\text{Library Size}))\}$  (<http://www.stanford.edu/group/nolan/screens/screens.html>). The number of infectious events per pool required for a saturated screen was determined to be 919, and 100,000 infectious events per pool were aimed for to increase chances of detecting true positives. This was done for two reasons. Firstly, some constructs might have been represented in the library at a lower frequency, and increasing infectious events would greatly increase the chances of assaying and detecting under-represented constructs. Secondly, as seen with E1A and E2F-DB (Figure 4.1), some constructs might only achieve abrogation of the growth arrest in a proportion of the cells that carry the construct; therefore increasing the number of infectious events could increase the chance of such constructs being detected by infecting more cells with these constructs.

Even though sensitivity was tested before the screen was performed, and 100 times the number of infectious events required per pool to achieve a saturated screen was used, the screen was not fully saturated. This was evident because a p53 shRNA construct present in the library was not detected in the screen. Pool 82 contained the p53 shRNA V2HS\_93615 construct, and as it had previously been demonstrated that knock-down of p53 rescues EcoR Clone 7 cells from the conditional growth arrest (see Figure 4.1 B), this construct should have been detected had the screen been saturating. Detection of the p53 shRNA would have been an internal control for the screen being saturating. However, this specific construct had never been tested and knock-down might not be very efficient. The same hairpin in the pGIPZ vector has now been tested for rescue from the conditional growth arrest in the EcoR Clone 7 cells, and it was found that at a low MOI (approx. 0.1 to 0.3) rescue was not very efficient, with less than 10 very small colonies forming from 10,000 seeded cells. At a higher MOI of 5 rescue by the construct was slightly improved, but still very weak (Figure 6.6). This suggests that multiple inserts might be required to provide sufficient knock-down to achieve rescue

with this construct, and even at a high MOI it only rescues a low percentage of EcoR Clone 7 cells. Therefore the construct might not be easily detected in a screen.

Another indication that the screen was not saturated is the fact that multiple shRNAs targeting the true positive hits Rab23 and IRF-1 were shown to achieve abrogation of the growth arrest (Chapter 7.1.3 and Chapter 7.1.5 respectively), but these constructs were not detected in the primary screen.

### **5.3.2 Stringency and false positives**

As discussed above, in an attempt to achieve saturation, the screen was performed with approximately 100,000 infectious events per pool, making a total of approximately 10,000,000. In total, 110 clones were picked, 6 of which carried inserts which were shown to overcome the conditional growth arrest of EcoR Clone 7 cells (Rab23 was found in two clones). Therefore, 104 clones can be assumed to have been background. This is not a very high background level; considering the total amount of infectious events, 0.00104% of infected cells gave false positives and continued to grow at the non-permissive temperature. The background level observed in cultures infected with the pools was comparable to that in lamin control-infected cultures.

Interestingly, in pools that were reseeded several hairpins were detected in multiple clones (probably from the same infectious event) but did not hold up in the secondary screen. In pool 3, the GLS2 shRNA V2HS\_71051 was detected seven times and the CLEC5A shRNA V2HS\_71374 five times, in pool 5 the sequence CCCTCAGCGTCTCCAATTACAT (it is unknown what gene it was designed to target; was not found in the database) seven times, in pool 11 the OXGR shRNA V2HS\_58850 twice, and in pool 20 the DSC2 shRNA V2HS\_62007 three times. This might have been caused by insertional mutagenesis. If so, primers could have been designed to sequence the area around the insert and identify the gene which was affected by insertion of the hairpin. But as insertional mutagenesis frequency should be the same for all viruses and is insert-independent, therefore the same amount of it should be observed in all pools, but some pools had no growing colonies at all. On the other hand, pools that were reseeded had to be reseeded because their titer had been under-estimated and too many

surviving cells remained following puromycin selection. More infectious events were achieved in these pools, which could explain why insertional mutagenesis could have occurred in these pools and not all others.

It is also possible that some EcoR Clone 7 cells could have acquired mutations that allowed them to grow at the non-permissive temperature, and when such cells got infected with a hairpin and then pools got reseeded, multiples of the progeny of these mutated cells, some of which carried the same hairpin, possibly from one infectious event, continued to grow at the non-permissive temperature. When uninfected EcoR Clone 7 cells were incubated at the non-permissive temperature, similar background levels were observed as in lamin control-infected cells. This supports the hypothesis that a certain percentage of cells, irrespective of infection with a virus, overcome the conditional arrest. It would be interesting to isolate such cells and analyse gene expression changes, comparing the expression profile of the cells that overcame the arrest to cells which arrested at the non-permissive temperature.

Another possibility is that some cells had a certain disposition/epigenetic change to overcoming the growth arrest, e.g. the p53 pathway was already partly inactivated. By infection of such cells with the hairpins identified in multiple clones, the additional knock-down of these genes could have led to such cells overcoming the arrest. These hairpins might only abrogate the growth arrest in the context of such epigenetic changes. However, if such mutations occurred which gave EcoR Clone 7 cells a disposition to overcoming the growth arrest and thereby led to detection of some target shRNAs, one would expect signal slightly above background when these targets were tested in a secondary screen. To avoid the occurrence of mutations, instead of growing cells in culture, cells were frozen down in bulk at passage 12/13 and infected at passage 14, and a fresh vial was used for each experiment. Cells from the same batch were used for the primary and secondary screen.

### **5.3.3 Screening in a p16<sup>INK4a</sup> knock-down background**

Berns and colleagues (Berns et al., 2004) conditionally immortalised fibroblasts analogous to the 226L 8/13 cells to screen for mediators of cellular senescence. They

found that knock-down of p16<sup>INK4a</sup> was insufficient to overcome the conditional growth arrest in their cells, but that knock down of p16<sup>INK4a</sup> in addition to knock-down of p53 gave stronger abrogation of the growth arrest compared to p53 knock-down alone. Therefore, they decided to screen for shRNAs that could abrogate the growth arrest in cells in which p16<sup>INK4a</sup> had been knocked down to facilitate the detection of mediators of senescence in their cell system.

It could have been established if knock-down of p16<sup>INK4a</sup> does not abrogate the growth arrest by itself and if it improves abrogation of the growth arrest achieved by p53 shRNA when introduced in combination into EcoR Clone 7 cells. If so, a similar approach as that of Berns and colleagues could have been used to facilitate the detection of mediators of senescence in EcoR Clone 7 cells.

#### **5.3.4 Secondary screen**

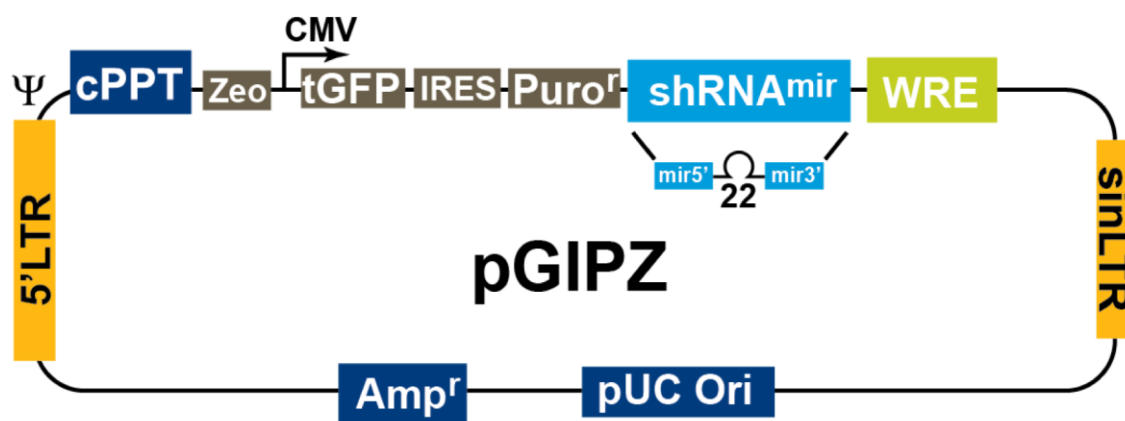
shRNAs that were detected in the primary pSM2c screen were ordered from the pGIPZ library and tested in a secondary screen, but knock-down of targets that failed in the secondary screen was never demonstrated. Even though in most cases the exact shRNA that had been isolated was tested in the secondary screen, it is possible that the level of expression of the shRNAs was lower from the pGIPZ vector system than from the pSM2c vector system, and that some true positive hits were therefore missed. To rule this out, knock-down by the shRNAs tested in the secondary screen should have been demonstrated, but this would have been very time consuming and expensive. Furthermore, it would most likely not have led to identification of additional true positives, as pGIPZ constructs were used under optimized conditions to achieve the best possible knock-down, such as 6 µg/ml puromycin and a high MOI.

In conclusion, 5 shRNAs which abrogate the EcoR Clone 7 conditional arrest were identified from the pSM2c library screen. To determine if abrogation of the growth arrest was caused by knock-down of the genes which these hairpins were designed to knock down was still unclear, off-target effects needed to be ruled out and knock-down of target genes needed to be demonstrated. This was further investigated and will be discussed in Chapter 7.



## **6 Results – pGIPZ primary and secondary shRNAmir screen**

The screen performed using version 3 of the Cold Spring Harbor Laboratories' pShagMagic2c (pSM2c) library targeted only 9,392 genes out of the total number of unique human genes, which is estimated to be 22,500. Therefore, when we got access to the Expression Arrest whole genome pGIPZ human lentiviral shRNAmir library from Thermo Scientific Open Biosystems, it was decided to screen this library for rescue in the EcoR Clone 7 cells. This library was developed in collaboration with Dr. Greg Hannon (CSHL) and Dr. Steve Elledge (Harvard). Like the pSM2c library, this library uses the design advantages of microRNA-adapted shRNA (shRNAmir), as shRNAs have been designed to mimic a natural microRNA primary transcript and are based on mir-30 by replacing the mature microRNA sequence in mir-30 with gene specific duplexes (Figure 5.3). As a vector backbone, the pGIPZ lentiviral vector was used (Figure 6.1). GFP, puromycin resistance and the shRNAs are all under the control of the CMV promoter, an RNA polymerase II promoter, as a polycistronic transcript, allowing visual marking and selection for shRNA expression.



Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
turbo GFP	Marker to track shRNAmir expression
Puro <sup>r</sup>	Mammalian selectable marker
AMP <sup>r</sup>	Ampicillin bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance in <i>E.coli</i>
SIN-LTR	3' Self inactivating long terminal repeat
RRE	Rev response element
ZEO <sup>r</sup>	Bacterial selectable marker

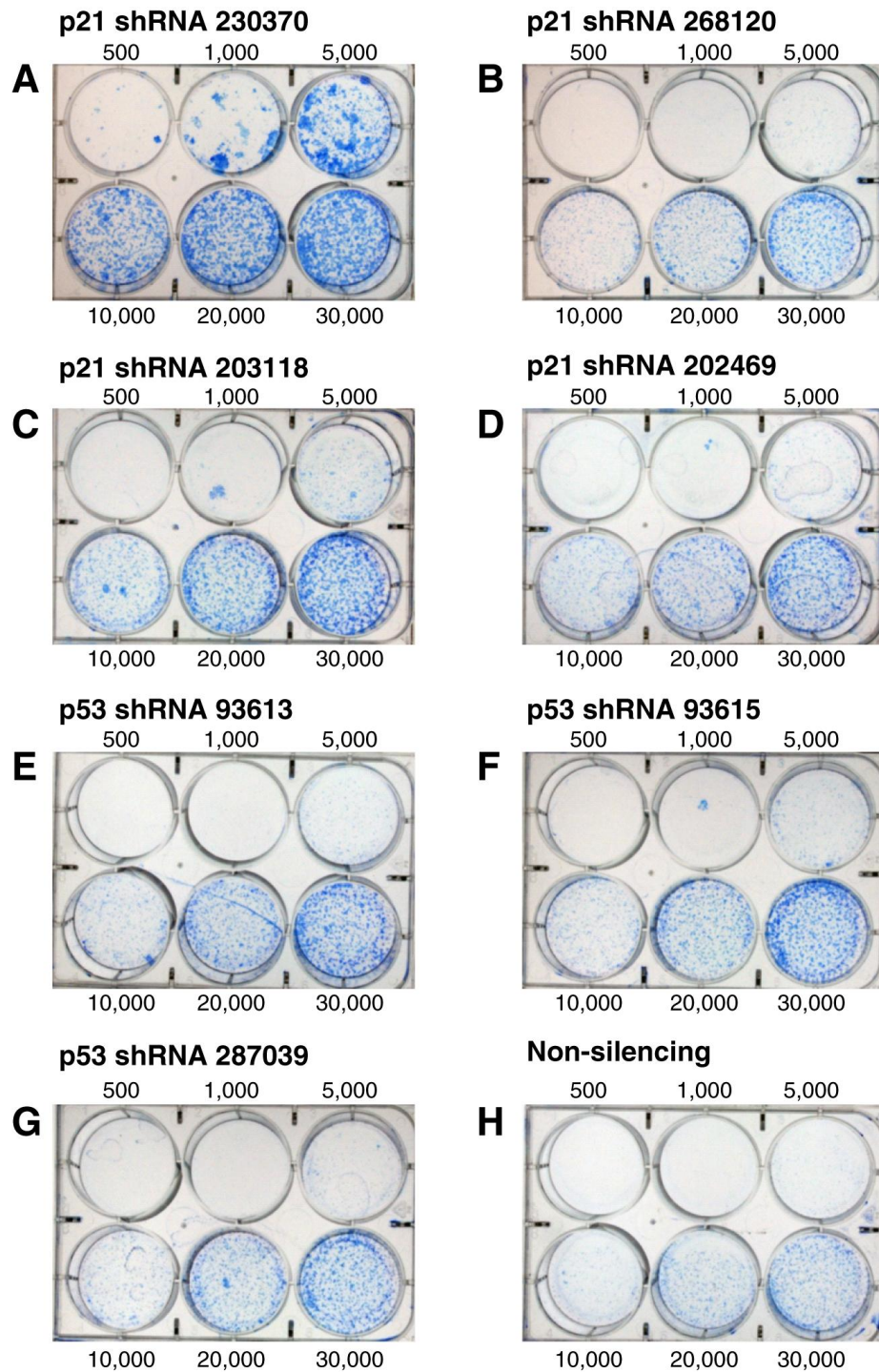
**Figure 6.1: pGIPZ vector design**

The pGIPZ vector carries GFP, puromycin resistance and the shRNAs under the control of the CMV promoter (RNA polymerase II promoter) as a single monocistronic transcript, allowing visual marking and selection for shRNA expression ("GIPZ lentiviral shRNAmir" - Thermo Scientific Open Biosystems: <https://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/>).

## **6.1 pGIPZ library optimisation**

### **6.1.1 pGIPZ p21 and p53 shRNA constructs were unable to abrogate the EcoR Clone 7 temperature-induced growth arrest when tested in the standard complementation assay**

Previously it had been demonstrated that the retroviral constructs containing hairpins which target p53 and p21, pRetroSuper-p53 and pRetroSuper-p21, were sufficient to rescue 226L 8/13 and EcoR Clone 7 cells from the conditional growth arrest at the non-permissive temperature (see Figure 3.5 and 4.1). Therefore, all Expression Arrest pGIPZ constructs targeting p53 (93613, 93615 and 287039) and p21 (230370, 202469, 268120 and 203118) and the non-silencing negative control construct (scrambled) were tested in the EcoR Clone 7 complementation assay. 1.5µg of each DNA were packaged as lentiviruses using HEK 293 cells and 1µg each of the packaging vectors. EcoR Clone7 cells were seeded at 500,000 cells in T75 flasks and the following day infected with 5ml virus over night in the presence of 15ml complete medium and 8 µg/ml polybrene. Selection was commenced 48 hour post-infection with 2µg/ml puromycin, and following drug selection for 5 days cells were reseeded at 500, 1,000, 5,000, 10,000, 20,000 and 30,000 cells per well in 6 well plates and shifted to the non-permissive temperature the following day. Surprisingly, none of the constructs seemed sufficient to rescue cells from the conditional growth arrest as all cultures appeared to be arrested within 5 days at the non-permissive temperature when examined under an inverted light microscope. Only with the construct V2LHS\_230370 some growing cells were observed at this point, but most of these also arrested within 7 days at the 38°C. When cells were stained following 3 weeks of incubation at the non-permissive temperature, most plates contained no colonies of growing cells and looked similar to the non-silencing negative control-infected plates (Figure 6.2). In wells in which 10,000 cells or more had been plated, blue stained cells were visible, but all of these were arrested. Colonies were detected in cells infected with the p21 shRNA V2LHS\_230370, and even though most of these comprised arrested cells, some growing colonies were present. Therefore this construct was used to optimise the assay for the pGIPZ library.



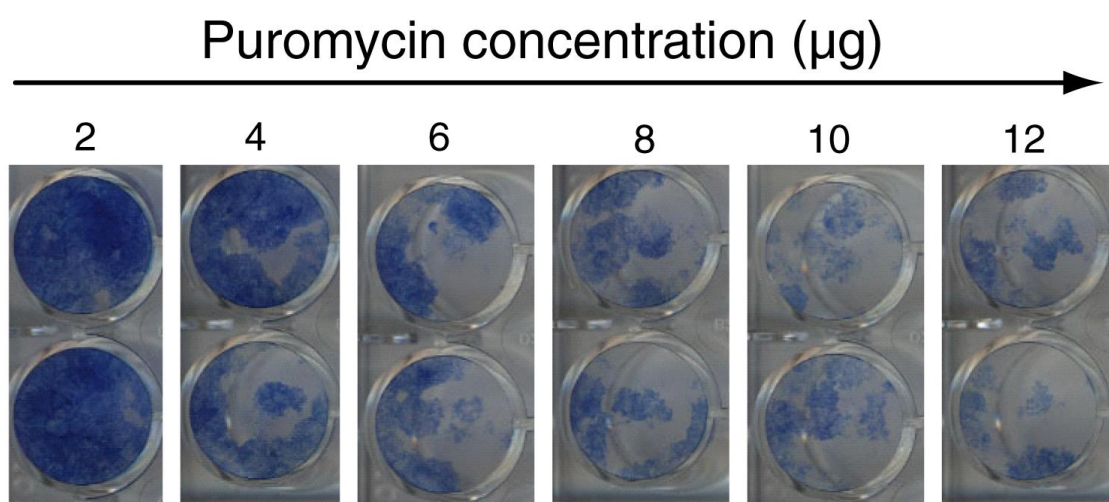
**Figure 6.2: pGIPZ p53 and p21 shRNA constructs complementation**

All Expression Arrest pGIPZ constructs targeting p53 (93613, 93615 and 287039) and p21 (230370, 202469, 268120 and 203118) and the non-silencing negative control construct (scrambled) were tested in the EcoR Clone 7 complementation assay. Apart from low levels of rescue with the p21 shRNA (**A**) 230370, none of the constructs were sufficient to abrogate the conditional arrest.

### **6.1.2 Puromycin selection optimisation - increased stringency: 6 µg/ml puromycin selection and lower cell density during drug selection improves abrogation of the conditional arrest**

How strong the knock-down by an shRNA is depends not only on its knock-down efficacy, but also on transfection efficiency, i.e. amount of siRNA introduced into cells (Hannon and Rossi, 2004). In the pGIPZ vector, the shRNA<sub>mir</sub> and the puromycin resistance are expressed under the same promoter. Therefore, increasing selection stringency to enrich for cells expressing puromycin resistance more strongly could potentially select for cells which express the shRNA at a higher level, thereby selecting for cells with greater knock-down. This theory was tested in the EcoR Clone 7 cells, using the p21 shRNA V2LHS\_230370 and the p53 shRNA V2LHS\_93615. These constructs were selected because in the complementation assay 230370 gave the most promising result as some growing colonies were observed, and 93615 seemed to have the best potential of the p53 shRNAs as some growth, even though very little, was also observed with 93615 (see Chapter 6.1.1).

First, it was determined if selection with higher concentrations of puromycin would lead to less cells surviving selection. Therefore, EcoR Clone 7 cells were seeded at 13,000 cells in 24-well plates and the following day infected with 1µl of dilutions of p21 shRNA V2LHS\_230370 lentivirus, ranging from 1 in 100 to 1 in 10,000,000. 48 hours post-infection puromycin selection was added, at 2, 4, 6, 8, 10 and 12 µg/ml. Cells were under selection for 5 days and grown for 9 days following drug selection. Cells were fixed and stained with methylene blue. At the dilutions ranging from 1 in 10,000,000 to 1 in 10,000 no colonies of cells were visible at any concentration of puromycin. At the lowest viral dilution, 1 in 100, growing cells were detected at all drug concentrations, but there was a clear difference in cell density between the three lowest puromycin concentrations, with most surviving cells at 2µg/ml, which was reduced at 4µ/ml and further reduced at 6 µg/ml (Figure 6.3). The result at 8 µg/ml puromycin looked similar to 6 µg puromycin, but colonies looked slightly less dense. As the difference of increasing concentration above 6 µg seemed to make little difference, but increasing puromycin selection from 2 to 6 µg led to a clear reduction in surviving cells, the effect of increasing concentration to 6 µg in the growth complementation assay was assessed.



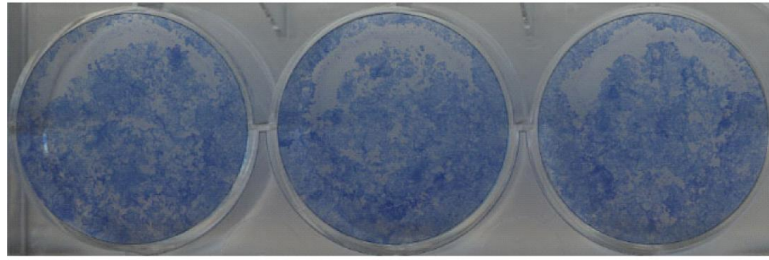
**Figure 6.3: Increasing puromycin concentration**

EcoR Clone 7 cells were seeded at 13,000 cells in 24-well plates and the following day infected with 1  $\mu\text{l}$  of p21 shRNA V2LHS\_230370 lentivirus diluted 1 in 100. Cells were selected at 2, 4, 6, 8, 10 and 12  $\mu\text{g/ml}$  puromycin, and then fixed and stained with methylene blue. Growing cells were detected at all drug concentrations, but there was a clear difference in cell density between the three lowest puromycin concentrations, with most surviving cells at 2  $\mu\text{g/ml}$ , which was reduced at 4  $\mu\text{g/ml}$  and further reduced at 6  $\mu\text{g/ml}$ . The result at 8  $\mu\text{g/ml}$  puromycin looked similar to 6  $\mu\text{g}$  puromycin, but colonies looked slightly less dense.

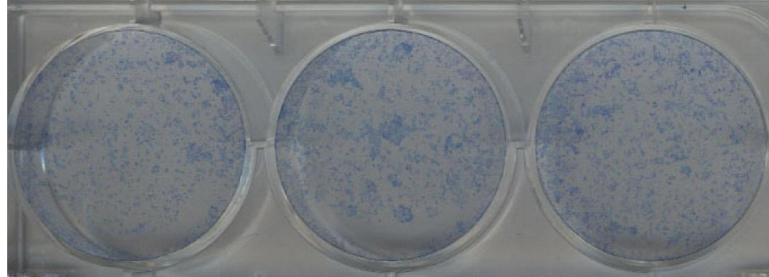
Non-silencing control vector, V2LHS\_230370 and V2LHS\_93615 were packaged and used to infect EcoR Clone7 cells. It had previously been shown that survival in puromycin is a function of cell density, and that puromycin selection is less efficient at higher densities (Cass, 1972). With this in mind, to achieve a lower cell density at the time of drug selection, cells were seeded at 250,000 cells per T75 flask the day before infection, reduced from 500,000 cells. 24 hours post-infection 6 µg/ml puromycin was added to medium, and cells were kept under selection for 5 days. Following drug selection, cells were reseeded at 10,000 cells in 6-well plates, which had previously been shown to be the ideal cell number for the EcoR Clone 7 complementation assay (Chapter 4.2), and shifted to the non-permissive temperature the following day. Following 2 weeks at the non-permissive temperature, cells were fixed and stained with methylene blue (Figure 6.4). No growing cells were detected in the non-silencing control-infected cells. A monolayer of growing cells was observed in cultures infected with the p21 shRNA 230370, but the p53 shRNA 93615 was still not sufficient to overcome the conditional growth arrest. Some larger colonies of arrested cells were visible, which when monitored during incubation at the non-permissive temperature continued to grow very slowly for about a week or so, after which they arrested. Therefore, slightly more blue staining was visible in these cultures as compared to the non-silencing negative control-infected cultures. This demonstrated that increasing stringency of puromycin selection would improve results achieved in the complementation assay with the p21 shRNA 230370, but not with the p53 shRNA 93615. Possibly, knock-down efficacy of this p53 shRNA was very low, so that selecting for higher expression was not sufficient to improve knock-down, whereas knock-down efficacy of the p21 construct 230370 was higher, therefore selecting for higher expression was sufficient to improve bypass of the growth arrest.



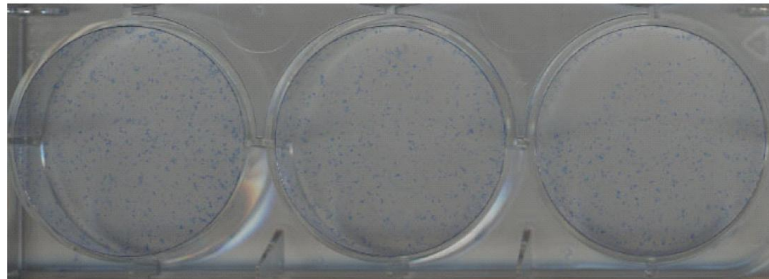
**p21 shRNA 230370**



**p53 shRNA 93615**



**Non-silencing**



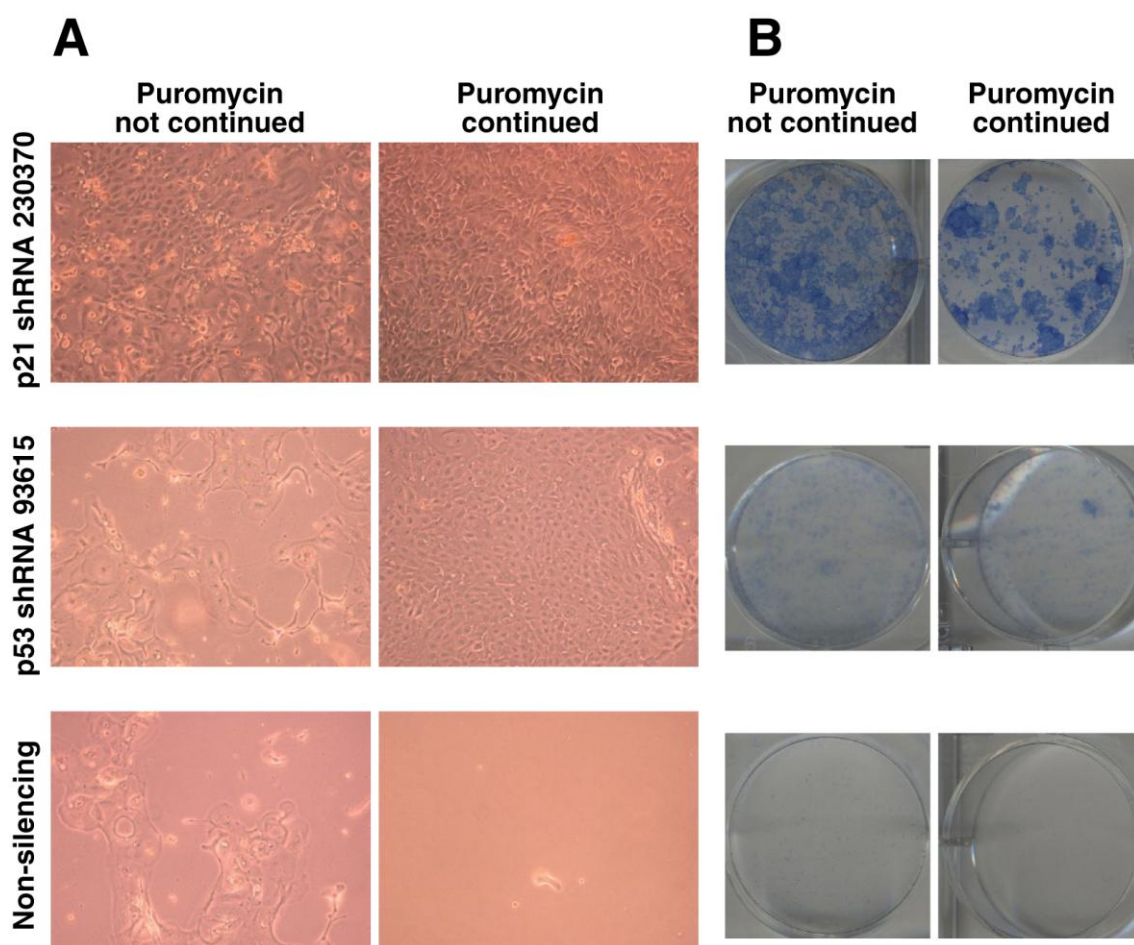
**Figure 6.4: Increased selection stringency improves rescue**

EcoR Clone 7 cells were seeded at 250,000 cells per T75 flask the day before infection, reduced from 500,000 cells, and infected with p21 shRNA 230370, p53 shRNA 93615, and non-silencing control. Cells were selected with 6  $\mu\text{g/ml}$  puromycin, and following drug selection, cells were reseeded at 10,000 cells in 6-well plates, and tested in the EcoR Clone 7 complementation assay. Following 2 weeks at the non-permissive temperature, cells were fixed and stained with methylene blue. A monolayer of growing cells was observed in cultures infected with the p21 shRNA 230370, but the p53 shRNA 93615 was still not sufficient to overcome the conditional growth arrest. No growing cells were detected in the non-silencing control-infected cells.



In addition to increasing puromycin concentration, the effect of continuous puromycin selection throughout the growth assay was tested. This was done because of the observation that even in growing p21 230370 knock-down cultures multiple arrested cells were visible, with some colonies arresting after more than a week of growth at the non-permissive temperature. It was possible that such cells somehow lost the insert or expression of the insert. If so, these cells would also lose puromycin resistance and not survive if selection was continued throughout the assay. To test this hypothesis non-silencing control, p21 shRNA 230370 and p53 shRNA 93615 constructs were packaged and used to infect EcoR Clone 7 cells. 24 hours post-infection 6 µg/ml puromycin were added to growth medium, and following 5 days of selection cells were reseeded at 10,000 cells/well in 6-well plates and the following day shifted to the non-permissive temperature. Cultures were then incubated at the non-permissive temperature for 2 weeks, and drug selection was either continued at 6 µg/ml, or it was removed when cells were reseeded. Following incubation at the non-permissive temperature, cells were photographed and then fixed and stained with methylene blue. Figure 6.5 A shows the effect of continued selection in the complementation assay at the non-permissive temperature on cell morphology. The first panel shows cells infected with the p21 shRNA 230370. Without selection at the non-permissive temperature many enlarged vacuole rich cells were visible in the culture, which were most likely arrested cells. If selection was continued, only healthy looking growing cells were observed. Only one growing colony was observed in p53 shRNA 93615 infected cells, which was in the culture in which puromycin selection was continued at the non-permissive temperature. But generally, in p53 shRNA 93615 infected cells no difference was detected between continued or discontinued selection; under both conditions many enlarged arrested looking cells were visible. In non-silencing control-infected cultures which were grown without puromycin multiple enlarged senescent looking cells were visible in the culture, but in cultures where puromycin selection was continued at the non-permissive temperature most cells had died. Methylene blue staining of these cultures (Figure 6.5 B) showed that p21 shRNA infected cells that were not kept under continuous selection had formed a near-monolayer, but this monolayer contained some arrested cells, whereas cells that were kept under continuous selection were visible as distinct dense colonies. No clear difference was visible between the two conditions in p53 shRNA

infected cells, but there was a clear difference with non-silencing control-infected cells as almost all cells had died in this culture (Figure 6.5 B). This shows that continued selection can potentially remove arrested cells which have lost expression of the insert, but as it removed almost all cells in the negative control culture it was not appropriate for the complementation assay, as this assay detects genes involved in senescence, not cell death.



**Figure 6.5: Continuous puromycin selection throughout the growth assay**

Continued drug-selection was tested in the complementation assay on cells infected with the p21 shRNA 230370, the p53 shRNA 93615, and non-silencing control. **(A)** Without selection at the non-permissive temperature in p21 shRNA 230370 infected cells many enlarged vacuole rich cells were visible in the culture. If selection was continued, only healthy looking growing cells were observed. In p53 shRNA 93615 infected cells no difference was detected between continued or discontinued selection; under both conditions many enlarged arrested looking cells were visible. In non-silencing infected cultures which were grown without puromycin multiple enlarged senescent looking cells were visible in the culture, but in cultures where puromycin selection was continued at the non-permissive temperature most cells had died.

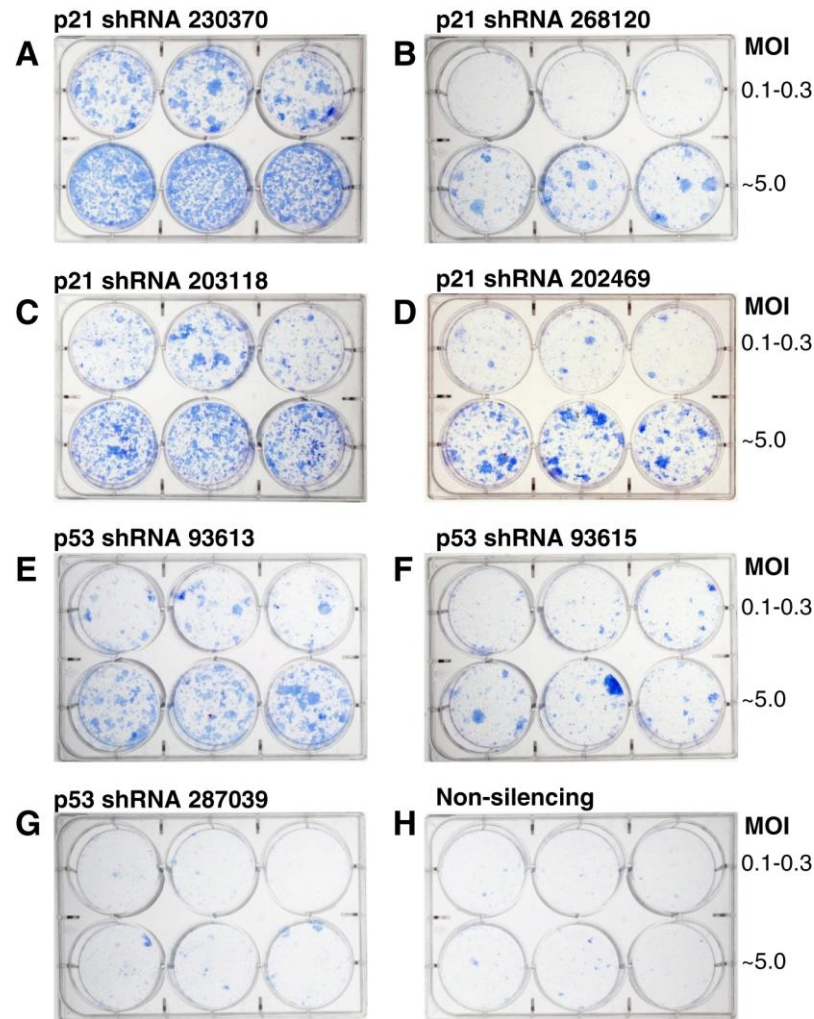
**(B)** Methylene blue staining of these cultures showed that p21 shRNA infected cells that were not kept under continuous selection had formed a near-monolayer, but this monolayer contained some arrested cells, whereas cells that were kept under continuous selection were visible as distinct dense colonies. No clear difference was visible between the two conditions in p53 shRNA infected cells, but there was a clear difference with non-silencing control-infected cells as almost all cells had died in culture with puromycin.

### **6.1.3 Increasing multiplicity of infection improves abrogation of the conditional arrest with pGIPZ p21 and p53 shRNAs**

The demonstration that more stringent puromycin selection could improve abrogation of growth arrest in the complementation assay with the p21 shRNA 230370 suggested that higher expression of the shRNA could improve knock-down. Higher expression can either be due to insertion of the insert into areas of the genome where it will be expressed at a higher level (e.g. more accessible to the cell's transcription machinery), or to multiple integrated copies of the insert. To detect targets in the screen, single inserts must be able to produce sufficient knock-down to achieve rescue from the conditional growth arrest. Open Biosystems recommends that the screen should be carried out with an MOI of 0.1 to 0.3 to achieve single inserts which can be identified by PCR amplification and sequencing. Therefore, to test if single inserts and stringent puromycin selection (i.e. 6 µg at lower cell density, see above) would lead to strong enough knock-down to rescue EcoR Clone 7 cells from the conditional arrest, EcoR Clone 7 cells were seeded at 250,000 cells in T75 flasks and the following day infected at two different MOIs, a low MOI of 0.1 to 0.3 and a higher MOI of approximately 5. All available shRNAs targeting p53 (93613, 93615 and 287039) and p21 (230370, 202469, 268120 and 203118) were tested, and the non-silencing control construct was used as the negative control. 24 hours post-infection selection with 6 µg/ml puromycin was started and following 5 days of selection cells were reseeded at 10,000 cells per well in 6-well plates and shifted to the non-permissive temperature the following day. Plates were incubated at the non-permissive temperature for two weeks and then fixed and stained with methylene blue (Figure 6.6). Very low background levels were detected in non-silencing control-infected cultures at the high and the low MOI. Similarly, hardly any colonies of growing cells were detected in cultures infected at a high or low MOI with the p53 shRNA construct 287039. In cultures infected at a low MOI with the p53 shRNA 93615 a few growing clones were detected, but infection at a high MOI led to a slight improvement of rescue from the conditional arrest, as in this condition several healthy looking colonies of growing cells were detected. Several colonies were detected in wells containing cells infected at a low MOI with the p53 shRNA 93613, but this was greatly improved when using a high MOI, in which case many growing colonies were visible. Similar results were observed for the p21 shRNAs

202469 and 268120, with moderate numbers of growing colonies visible at a low MOI, but many more at a high MOI. The p21 shRNA 230370 achieved high levels of rescue even at a low MOI, which was improved at a higher MOI. Similarly, the p21 shRNA 203118 produced good levels of rescue even at a low MOI, but rescue was significantly improved when cells were infected at a high MOI. Furthermore it might be of interest to note that when cultures were reseeded, pellets of cells infected at high MOIs were visibly green, presumably because of GFP expressed from the inserts, whereas cultures infected at low MOIs did not appear green. When reseeded cultures were observed under a fluorescent microscope, levels of GFP expression were much higher in cultures infected at an MOI of 5 than at an MOI of 0.1 to 0.3.

In summary, increasing the MOI from 0.1 – 0.3 to the higher MOI of 5 improved abrogation of growth arrest achieved by the shRNAs in the complementation assay.



**Figure 6.6: The effect of MOI on abrogation of growth arrest achieved by the pGIPZ p21 and p53 shRNAs in the complementation assay**

The pGIPZ shRNAs targeting p53 (93613, 93615 and 287039) and p21 (230370, 202469, 268120 and 203118) were tested in the complementation assay at a multiplicity of infection of approximately 0.1-0.3 and 5, with puromycin selection with 6  $\mu\text{g}/\text{ml}$ . **(A)** 230370 was sufficient to abrogate at both MOIs, but more abrogation was improved at the higher MOI. **(B)** With 268120, only very low levels of growth were achieved at the lower MOI, but this was significantly improved at the higher MOI. **(C)** 203118 was sufficient to abrogate at both MOIs, but abrogation was improved at the higher MOI. **(D)** 202469 achieved low levels of growth at the low MOI, which was significantly improved at the higher MOI. **(E)** 93613 achieved low levels of growth at the low MOI, which was significantly improved at the higher MOI. **(F)** 93615 achieved low levels of growth at the low MOI, which was only slightly improved at the higher MOI. **(G)** 287039 was insufficient to abrogate the growth arrest. **(H)** Very low levels of background were visible in the non-silencing control infected cultures.

#### ***6.1.3.1 Knock-down is improved by using a higher multiplicity of infection***

Following the finding that a higher MOI could improve rescue from the conditional growth arrest by several p21 and p53 shRNA constructs, knock-down of p21 at a high and low MOI with the p21 shRNA constructs was evaluated.

#### ***Western blotting***

For Western Blotting, T75 flasks were set up when cells were reseeded for the growth assay, and cultures were lysed using RIPA buffer when cells were about 80% confluent. Medium was changed the day before extraction. Protein concentration was determined using the Bradford assay, and 35µg of protein were loaded on a 16% gel with Kaleidoscope protein size marker. P21 antibody SX118 was used at a dilution of 1 in 250 in 5% milk PBS Tween at 4°C over night. Secondary anti-mouse antibody was used at 1 in 10,000 in 5% milk PBS Tween for one hour at room temperature. β-tubulin was used as a control for equal protein loading, and the non-silencing control was used as a negative control for p21 knock-down. The 21 kDa band that corresponded to p21 [as indicated in the literature (Yamamoto et al., 1998)] was strongly visible in the non-silencing control, but completely disappeared with the 230370 shRNA both at a low and high MOI (Figure 6.7 A). For the construct 268120 a decrease was observed in the band as compared to the non-silencing control at a low MOI, and this result was slightly improved at a high MOI. Results were similar but more striking for the construct 202469, where a low MOI reduced the band slightly, but at a high MOI it was strongly reduced. For the construct 203118, the band was strongly reduced at a low MOI, and hardly visible at a high MOI.

#### ***Real-time quantitative RT-PCR***

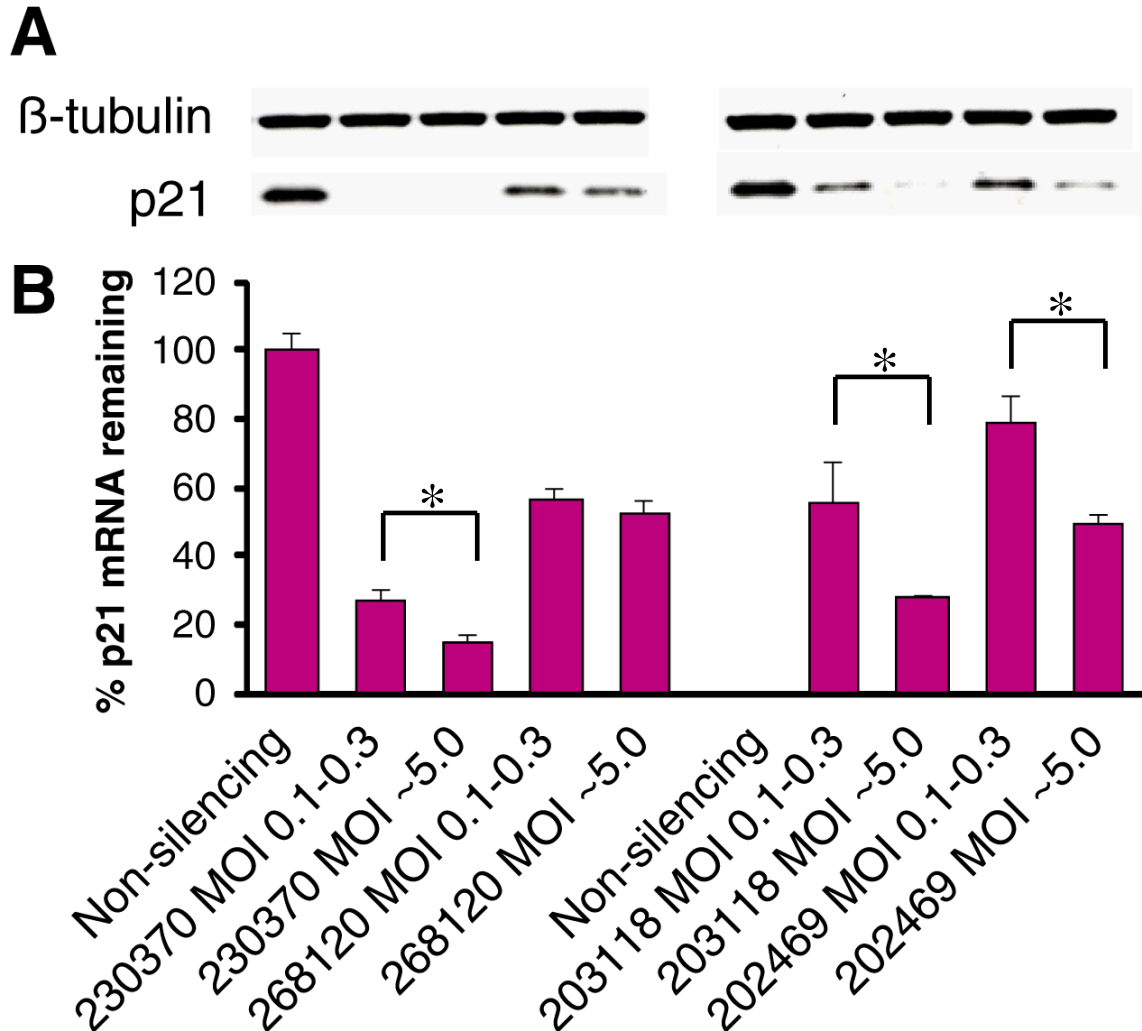
Real-time quantitative RT-PCR was performed using the Ambion Cells-to-CT kit and a p21 TaqMan Gene Expression Assay (containing primer sets and probes). Cells infected with the non-silencing control and the p21 shRNA constructs were seeded at 10,000 cells in a 96 well plate. The following day cells were lysed in the presence of DNase, which was followed by an RT reaction of the cell lysates. cDNA products were then used for the real-time PCR reaction. Figure 6.7 B shows the percentage of mRNA remaining in cells infected with the different p21 shRNA constructs as compared to the

total mRNA level in non-silencing control infected cells. The best knock-down was achieved by 230370, which also achieves the best abrogation of growth arrest in the complementation assay. The shRNA 230370 achieved knock-down to 27.9% at the low MOI, and at the high MOI knock-down to 15.7%, even though no band was observed by western blotting at either MOI. The construct 203118 achieved the second strongest knock-down, and it also produces the second strongest abrogation of growth arrest in the complementation assay. 203118 achieved knock-down to 56% when cells were infected at a low MOI, and knock-down to 28.8% in cultures infected at a high MOI. The other two constructs are the least efficient shRNAs, and also produce the least significant result in the complementation assay. The shRNA 268120 achieved knock-down to 57.5% at the low MOI, and 52.6% at the high MOI, which correlated with the western blot. With the shRNA 202469 knock-down to 79.5% was achieved at a low MOI, which was improved to 50.3% when cells were infected at a high MOI; again, this correlates with the results obtained by western blotting, apart from knock-down appearing slightly more efficient when analysed by western blotting. Generally, knock-down appeared slightly more efficient when observed by western blotting than by real-time quantitative RT-PCR, which could be due to the shRNAs ability to not only degrade the mRNA but also inhibit translation, or due to western blotting being less sensitive.

To summarise, increasing the MOI from 0.1/0.3 to an MOI of 5 improved knock-down of p21 achieved by the shRNA constructs. The increased knock-down of p21 by the individual shRNAs at the higher MOI was reflected by the complementation assay, where stronger abrogation of the conditional growth arrest was achieved by the higher MOI (Figure 6.6).

These results demonstrated that p21 shRNAs in the library had varying knock-down efficiency. The more efficient ones produced sufficient knock-down with single inserts to abrogate EcoR Clone 7 conditional growth arrest when selected with 6 µg/ml puromycin. Therefore, it should be possible to detect genes which can cause EcoR Clone 7 cells to overcome the arrest in the screen if efficient shRNAs are represented in the library.





**Figure 6.7: Knock-down is improved by using a higher multiplicity of infection**

(A) Western blotting and (B) quantitative RT PCR demonstrated that knock-down of p21 by the pGIPZ shRNAs 230370, 202469, 268120 and 203118 was improved by infection at MOI 5 compared to MOI 0.1-0.3. Bars represent normalised expression values relative to β-Actin. Error bars represent the standard error of the mean. \* indicates  $p \leq 0.05$ , Student's t-test, one-tailed distribution assuming equal variance.

#### **6.1.4 Conclusions of the optimisation for set-up of the screen**

To perform the screen, single inserts needed to be sufficient to achieve rescue from the conditional arrest. Infecting cells at an MOI of 5 was a good strategy when testing individual targets, but not for the screen as this strategy relied on improving knock-down by introducing several copies of the same hairpin into a cell, which would be unrealistic in a pooled screen. With stringent puromycin selection, abrogation of the growth arrest could be achieved even at an MOI of 0.1 to 0.3 with two of the p21 shRNA constructs and one of the p53 shRNA constructs. The above described results demonstrated that the pGIPZ shRNA<sub>mir</sub> library was a potentially useful tool to identify shRNA<sub>mir</sub>s which could overcome the EcoR Clone 7 conditional growth arrest.

To increase the likelihood of detecting shRNAs which could bypass senescence arrest, the amount of infectious events required for each pool to achieve 99% confidence that each construct in the pool was covered was calculated (Chapter 2.8.2), and this amount was increased five-fold for pools 1 and 2, and ten-fold for pools 3, 4, 5, 6 and 7. The titer for the pools was determined at 6 µg/ml puromycin selection to select for cells that express the insert at higher levels.

The frequency of the event which was being searched for needed to be considered to achieve sufficient sensitivity to detect positives in the pools. The library consisted of 7 pools, each containing approximately 9,000 constructs. Therefore, to test for sensitivity, a 1 in 9,000 mix of the p21 shRNA 230370 positive control DNA in non-silencing negative control DNA was made, packaged, and the viral supernatant was used to infect 2,500,000 cells at an MOI of 0.1 to achieve 250,000 infectious events. Non-silencing control virus was also used to infect 2,500,000 cells at an MOI of 0.1 to achieve 250,000 infectious events. Following 3 weeks of growth at the non-permissive temperature, 12 colonies were observed in cells infected with the mix, whereas only 3 were observed in non-silencing control infected cells, demonstrating that the complementation assay was sensitive enough to detect constructs in the pools which were able to abrogate the growth arrest (data not shown).

## 6.2 pGIPZ screen

### 6.2.1 Determination of the titer of the 7 pools in EcoR Clone 7 cells

The titer of these 7 pools in HEK 293 cells had been determined by Open Biosystems. To save the virus we received for each pool and also to minimise freeze-thawing of the virus pools as this would have reduced their titer (only one aliquot received for each pool), Open Biosystems supplied non-silencing control virus also titered in HEK 293 cells to determine the relative titer in our target cells compared to HEK 293 cells. The difference in titer of the lentivirus control between HEK 293 cells and EcoR Clone 7 cells was determined, and this comparison was used to determine the titer of each pool in EcoR Clone 7 cells.

The day before transduction, a 24-well tissue culture plate with EcoR Clone 7 cells was seeded at 13,000 cells per well. 4 replicates of eight dilutions of the non-silencing viral stock were made, each a series of 5-fold dilutions to reach a final dilution of 390625-fold, and cells transduced by adding 25 µl of diluted non-silencing control virus in the presence of 225 µl of serum free medium and 8 µg/ml polybrene. Following 4 hours of incubation wells were topped up with 1ml of complete E93 medium containing 8 µg/ml polybrene; medium was replaced with complete E93 the next morning. 24 hours post-infection selection was started at 6 µg/ml puromycin and including 5 days of selection cells were grown for 14 days, stained with methylene blue, and colonies were counted to determine the titer of the non-silencing control in the target cells. This was then used to determine the titer of the 7 pools in the EcoR Clone 7 cells with the following calculation:

Non-silencing control titer in HEK 293 cells ÷ non-silencing control titer in EcoR Clone 7 cells = **relative reduction in transduction efficiency**

Titer of pools in HEK 293 cells ÷ relative reduction in transduction efficiency = **titer of pools in EcoR Clone 7 cells**

Table 6.1 shows the results of the titrations and the amount of virus that was used to achieve the required MOI of 0.1 in the screen.

Pool	Titre in HEK 293 cells (TU/ml)	Relative reduction in transduction efficiency	Titre in EcoR Clone 7 cells (TU/ml)	Amount of virus used per 1,000,000 cells to achieve an MOI of 0.1
1	$6.0 \times 10^9$	25	$2.4 \times 10^8$	4.2 $\mu$ l
2	$4.3 \times 10^9$	25	$1.72 \times 10^8$	5.8 $\mu$ l
3	$5.15 \times 10^8$	25	$2.0 \times 10^7$	4.9 $\mu$ l
4	$1.47 \times 10^9$	25	$5.88 \times 10^7$	1.7 $\mu$ l
5	$1.05 \times 10^9$	25	$4.2 \times 10^7$	2.4 $\mu$ l
6	$1.41 \times 10^9$	25	$5.64 \times 10^7$	1.8 $\mu$ l
7	$2.0 \times 10^9$	25	$8.0 \times 10^7$	1.3 $\mu$ l

**Table 6.1: pGIPZ pool titers**

The titer and the amount of virus required to infect 1,000,000 cells at an MOI of 0.1 (i.e. the amount of virus required for 100,000 infectious events) was determined for each pool. A total of 25  $\mu$ l were available per pool.

### **6.2.2 Infection and growth conditions of EcoR Clone 7 cells, and identification of shRNA inserts in colonies of cells that overcame the conditional arrest**

At the time of the screen the library consisted of a total of about 62,000 constructs, which we received from Open Biosystems in 7 pools of lentivirus each containing approximately 9,000 constructs. As for the pSM2c screen, EcoR Clone 7 cells frozen in bulk at passage 12/13 were used for this screen; EcoR Clone 7 cells at passage 14 were infected with each pool at an MOI of 0.1. As explained in chapter 2.8.2, it was determined that 41,444 infectious events per pool were required to assay all the shRNA constructs in each pool at the 99% confidence level. This amount was increased approximately five-fold for pools 1 and 2 (250,000 infectious events), and ten-fold for pools 3, 4, 5, 6 and 7 (500,000 infectious events).

The day before transduction, for pools 1 and 2 five T75 flasks of 500,000 EcoR Clone 7 cells were seeded, and for pools 3-7 five T150 flasks of 1,000,000 EcoR Clone 7 cells were seeded. The next day medium was removed and the virus added to achieve an MOI of 0.1 and the total volume of liquid was made up to 10 ml in T75 or 15 ml in T150 flasks with serum-free E93 medium containing 8 µg/ml polybrene. Approximately 4-6 hours post-transduction, 10 ml per T75 and 15 ml per T175 of E93 medium with 20% FCS and 8 µg/ml polybrene was added to the cells and incubated overnight. The next morning medium was replaced with complete E93, and 24 hours post-transduction selection at 6 µg/ml puromycin was started, and after 5 days selection was discontinued and cultures were shifted to 38°C. Non-silencing control-transduced cells were used as the negative control, and p21 shRNA 230370 and 268120-transduced cells were used as the positive control. Cells were fed twice per week, and after two to three weeks of incubation at the non-permissive temperature clones that overcame the conditional arrest could be detected. Clones which were growing at the non-permissive temperature were grown to sufficient size and then ring-cloned and seeded in 24 well plates. They were grown to confluence and then split into 6 well plates where they were grown to confluence before DNA was extracted. 250-300ng genomic DNA was used in a PCR reaction with a forward and a reverse sequencing primer provided by Open Biosystems. PCR reactions were then resolved alongside 1kb+ DNA ladder on a 1.4% agarose gel to

check for the generation of 561 base pair PCR products that could be visualised on a UVP. The PCR product was purified, and DNA sequencing was carried out by the genetics group of the MRC Prion Unit using the sequencing primer provided by Open Biosystems.

### **6.3 pGIPZ Library Screen Hits**

A total of 22 candidate hairpins were identified from the 7 pools; this is summarised in Table 6.2.

#### **POOL 1**

Following drug selection cultures were shifted to the non-permissive temperature without being reseeded. No colonies of growing cells were detected in any of the flasks. To confirm that no colonies had been overlooked, flasks were stained with methylene blue. This confirmed that no growing colonies were present.

#### **POOL 2**

Pool 2 was not reseeded before cultures were shifted to the non-permissive temperature. Two colonies of growing cells were detected in 2 separate flasks. The inserts in these clones were identified as the LOC343425 shRNA *V2LHS\_25465* and the FLJ31301 (full-length long Japan 31301) shRNA *V2LHS\_68969*.

#### **POOL3**

Cultures were not reseeded before they were shifted to the non-permissive temperature. Two clones were picked from pool 3. The inserts *V2LHS\_256670*, designed to knock down SERPIND1 [serpin peptidase inhibitor, clade D (heparin cofactor), member 1] and *V2LHS\_127153*, designed to knock down LOC351347, were identified in these clones.

#### **POOL4**

As cells had become too confluent for the growth assay following drug selection, these cultures needed to be reseeded before they were shifted to the non-permissive temperature. After 3 weeks of incubation at the non-permissive temperature four very slow growing clones were picked, but they arrested after ring cloning.

#### **POOL5**

Cultures were slightly too confluent following drug selection and were therefore reseeded before they were shifted to the non-permissive temperature. 5 clones were picked from this pool, all of which carried the same two inserts. As one signal was stronger than the other, it was possible to separate the sequences and identify these inserts as the CCNL1 (Cyclin L1) shRNA **V2LHS\_40050** and the BRCA1 (breast cancer 1) shRNA **V2LHS\_254609**. Because pool had been reseeded the 5 clones most likely stemmed from the same cell which was infected by both inserts.

#### **POOL6**

Nine clones were picked from this pool, two of which carried multiple inserts which were not identified due to time limitations. Four inserts were identified as **V2LHS\_260630**, **V2LHS\_201625**, **V2LHS\_60002** and **V2LHS\_36376**. **V2LHS\_16814**, which targets FOXA1 (Forkhead box protein A1), was detected in three individual clones. As cultures had not been reseeded before they were shifted to the non-permissive temperature, these three clones were likely to originate from different cells infected with the insert, making it a very promising candidate.

#### **POOL7**

Although this pool was not reseeded, cells were slightly more confluent than desired for the EcoR Clone 7 growth assay. 16 colonies were picked, 5 of which contained multiple inserts and could not be identified due to time restraints. The other 11 colonies contained inserts which were identified as **V2LHS\_157053** (FLJ22531 shRNA), **V2LHS\_14455** [speckle-type POZ protein-like (SPOPL) shRNA], **V2LHS\_198522** [katanin p60 (ATPase-containing) subunit A 1 (KATNA1) shRNA], **V2LHS\_49974** [BMP and activin membrane-bound inhibitor homolog (BAMBI) shRNA], **V2LHS\_42783** [zinc finger protein 42 (ZNF754) shRNA], **V2LHS\_101637** (LOC150935 shRNA), **V2LHS\_70349**, **V2LHS\_275571**, **V2LHS\_138523** [ring finger protein 135 (RNF135) shRNA], **V2LHS\_52550** and **V2LHS\_117076**.

Pool	Gene name	shRNAs identified and tested
1	none	none
2	LOC343425	V2LHS_25465
	FLJ31301	V2LHS_68969
3	SERPIND1	V2LHS_256670
	LOC351347	V2LHS_127153
4	none	none
5	CCNL1	V2LHS_40050 also tested V2LHS_40051 and V2LHS_40054
	BRCA1	V2LHS_254609 also tested V2LHS_238842, V2LHS_90877, V2LHS_280394 and V2LHS_198913
6	FOXA1	V2LHS_16814
	FAH	V2LHS_260630
	FLJ90308	shRNA V2LHS_36376
	unknown	V2LHS_201625
	unknown	V2LHS_60002
7	SPOPL	V2LHS_14455
	FLJ22531	V2LHS_157053
	KATNA1	V2LHS_198522
	BAMBI	V2LHS_49974
	ZFP42	V2LHS_42783
	MGC13061	V2LHS_138523
	LOC150935	V2LHS_101637
	unknown	V2LHS_70349
	unknown	V2LHS_275571
	unknown	V2LHS_52550
	unknown	V2LHS_117076

**Table 6.2: pGIPZ primary screen and secondary screen summary**

This table summarizes candidate genes, specific shRNAs isolated in the screen and hairpins tested in the secondary screen. Highlighted in yellow are shRNAs validated in the secondary screen.



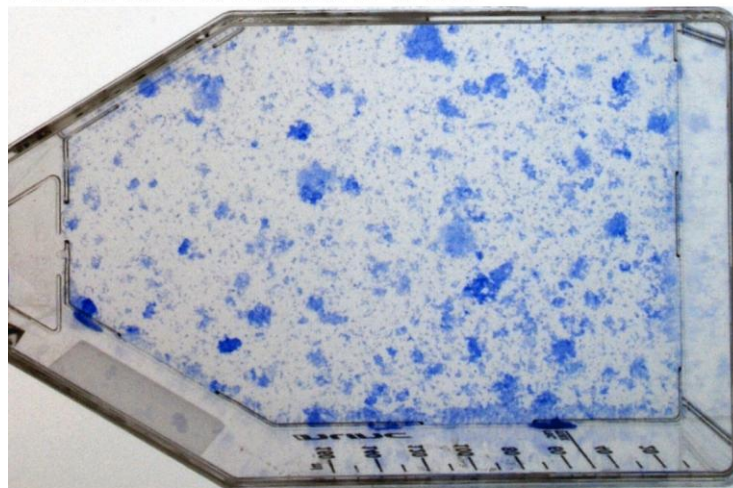
## 6.4 Secondary screen

A total of 22 candidate hairpins had been identified in the primary screen, and these candidates were tested in a secondary screen. In total, 28 shRNAs were tested in the secondary screen, targeting the 22 candidate genes. Table 6.2 summarizes candidate genes, specific shRNAs isolated in the screen and hairpins tested in the secondary screen. For validation in a secondary screen, the shRNAmirs that were identified were then obtained from the Open Biosystems lentiviral pGIPZ library and tested in the EcoR Clone 7 complementation assay. As for the primary screen, EcoR Clone 7 cells frozen in bulk at passage 12/13 were used for the validation screen. The complementation assay was used as described in section 3.5.1, with three modifications: Puromycin selection was started 24 hours instead of 48 hours post-infection at 6µg/ml instead of 2µg/ml. Also, cells were infected at a high MOI for the secondary screen (5 ml of virus per 80,000 cells), as a higher MOI was shown to produce better knock-down and rescue with the positive control shRNAs targeting p21, and better rescue with positive control shRNAs targeting p53 (Figure 6.6 and 6.7). pGIPZ non-silencing control (scrambled) was used as a negative control. Silencing of 7 genes led to EcoR Clone 7 cells overcoming the conditional arrest in the secondary screen.

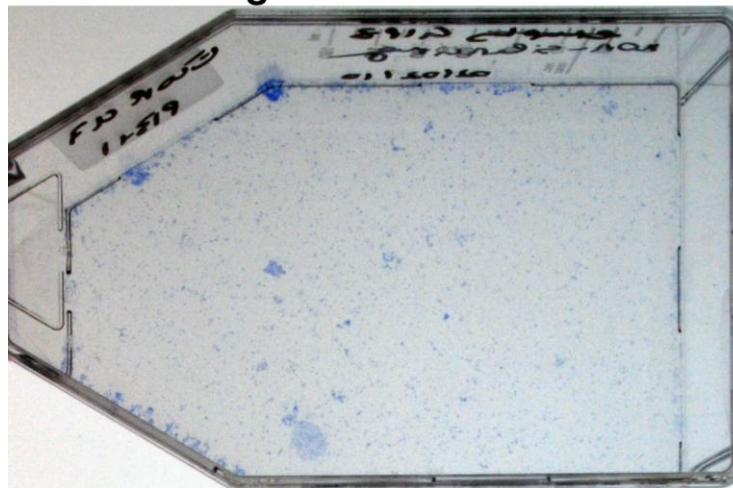
### V2LHS\_25465

This shRNA was isolated in pool 2. It was designed to knock-down LOC343425. V2LHS\_25465 was tested in the complementation assay. Following 2 weeks of incubation at the non-permissive temperature very few colonies were detected in the non-silencing control-infected cells, but multiple colonies of growing cells were detected in cultures infected with the shRNA. The experiment was set up in triplicate and representative images are shown in Figure 6.8. This showed that the shRNA 25465 was sufficient to abrogate the conditional growth arrest of EcoR Clone 7 cells.

### LOC343425 shRNA 25465



### Non-silencing



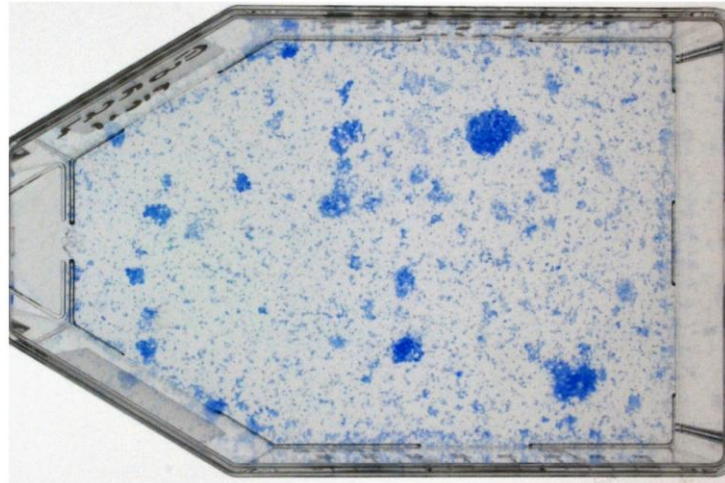
**Figure 6.8: V2LHS\_25465 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The LOC343425 shRNA construct V2LHS\_25465 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 25465, and only very low background levels were detected in cells infected with the non-silencing control virus.

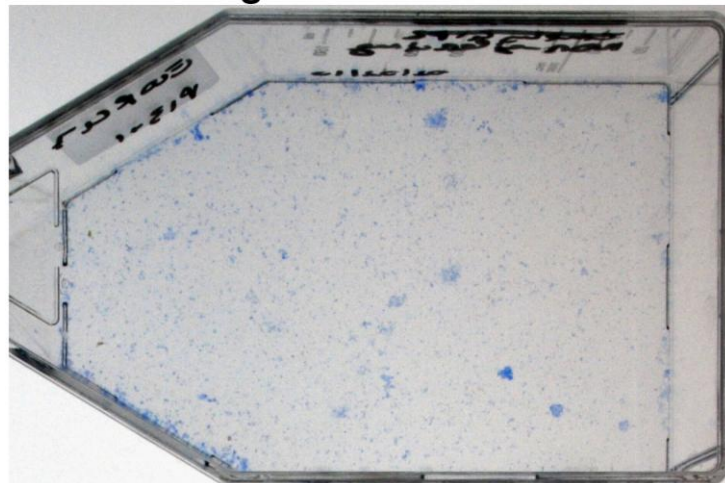
### **V2LHS\_68969**

The FLJ31301 shRNA V2LHS\_68969 was detected in pool 2. Following incubation at the non-permissive temperature, very low background levels were visible in cultures infected with the non-silencing control, whereas multiple colonies of growing cells were visible in cultures infected with V2LHS\_68969. The experiment was set up in triplicate and representative images are shown in Figure 6.9. This demonstrated that the shRNA 68969 was sufficient to overcome the temperature-shift-induced growth arrest of EcoR Clone 7 cells.

### FLJ31301 shRNA 68969



### Non-silencing



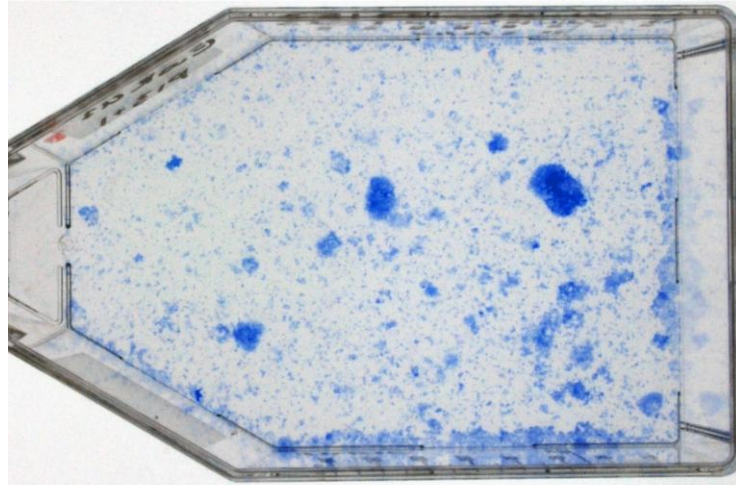
**Figure 6.9: V2LHS\_68969 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The FLJ31301 shRNA construct V2LHS\_68969 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 68969, and only very low background levels were detected in cells infected with the non-silencing control virus.

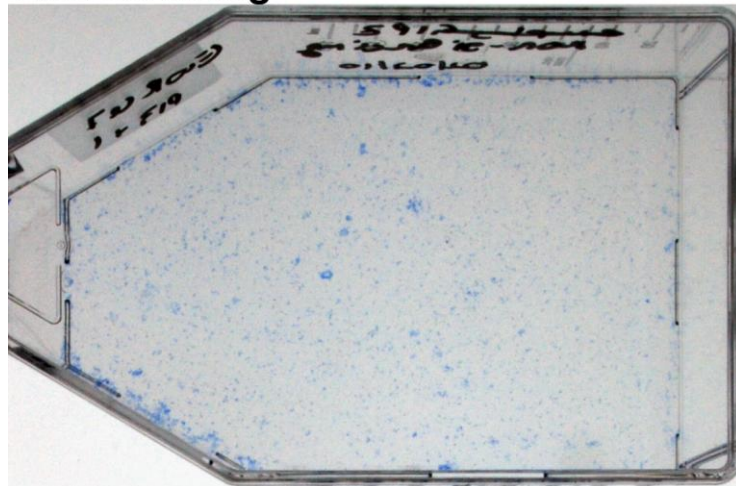
### **V2LHS\_127153**

The LOC351347 shRNA 127153 was detected in pool 3. It was tested in the EcoR Clone 7 complementation assay. Following incubation at the non-permissive temperature, only very low background levels were visible in non-silencing control-infected cultures, but multiple colonies of growing cells were detected in cultures infected with V2LHS\_127153. The experiment was set up in triplicate and representative images are shown in Figure 6.10. This demonstrated that the shRNA 127153 was sufficient to abrogate the conditional growth arrest of EcoR Clone 7 cells.

### LOC351347 shRNA 127153



### Non-silencing



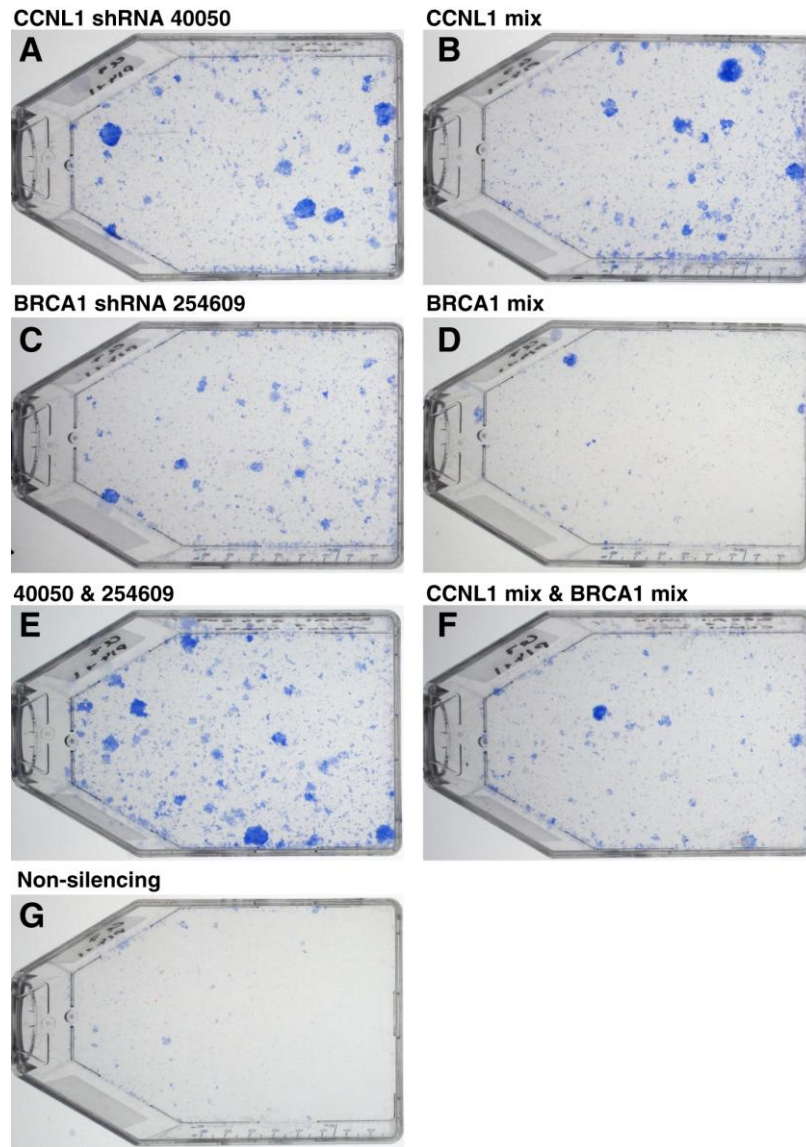
**Figure 6.10: V2LHS\_127153 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The LOC351347 shRNA construct V2LHS\_127153 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 127153, and only very low background levels were detected in cells infected with the non-silencing control virus.

## **V2LHS\_40050 AND V2LHS\_254609**

The CCNL1 shRNA V2LSH\_40050 and the BRCA1 shRNA V2LSH\_254609 were detected together in 5 clones derived from pool 5. Since these inserts were detected together, the CCNL1 hairpin 40050 and the BRCA1 hairpin 254069 were tested together and separately in the secondary screen. Also, a mix of two other CCNL1 hairpins, V2LHS\_40051 and V2LHS\_40054, and a mix of four further BRCA1 hairpins, namely, V2LHS\_238842, V2LHS\_90877, V2LHS\_280394 and V2LHS\_198913, were tested in a secondary screen. To make the mix, DNA was mixed 1:1 for the CCNL1 hairpins and 1:1:1:1 for the BRCA1 hairpins before it was packaged using HEK 293 cells. Cells were infected with 40050 and the CCNL1 mix, and with 254609 and the BRCA1 mix. Cells were also co-infected with the two hairpins detected in the screen 40050 and 254069, and co-infected with the CCNL1 and BRCA1 shRNA mixes. Only very low background levels were observed in non-silencing control-infected cells, whereas many colonies of growing cells were detected in cells infected with the shRNAs detected in the screen, CCNL1 shRNA 40050 and BRCA1 shRNA 254069 individually. The experiment was set up in triplicate and representative images are shown in Figure 6.11. Many colonies were also detected in cells co-infected with both these shRNAs; there were slightly more colonies when cells were co-infected. In cultures infected with the mix of the two CCNL1 shRNAs 40051 and 40054 many colonies of growing cells were visible, but hardly any colonies of growing cells were visible in cells infected with the BRCA1 shRNA mix (which did not contain the hairpin 254069). In cultures co-infected with the CCNL1 mix and the BRCA1 mix growing colonies were detected, but less than in cultures co-infected with the two hairpins detected in the screen.





**Figure 6.11: V2LHS\_40050 & V2LHS\_254609 are sufficient to abrogate the EcoR Clone 7 growth arrest**

The CCNL1 shRNA 40050, and mix of the CCNL1 shRNAs 40051 and 40054, and the BRCA1 shRNA 254609 and a mix of the BRCA1 shRNAs 238842, 90877, 280394 and 198913 were tested in the complementation assay. (A) The CCNL1 shRNA 40050 and (B) the CCNL1 mix were sufficient to abrogate the EcoR Clone 7 conditional arrest. (C) The BRCA1 shRNA 254609 was sufficient, but (D) the BRCA1 mix was insufficient to abrogate the EcoR Clone 7 conditional arrest. (E) Co-infection with the two hairpins detected in the screen 40050 and 254069, and (F) co-infection with the CCNL1 and BRCA1 shRNA mixes was sufficient to abrogate the growth arrest. (G) Only very low background levels were observed in non-silencing control-infected cells.

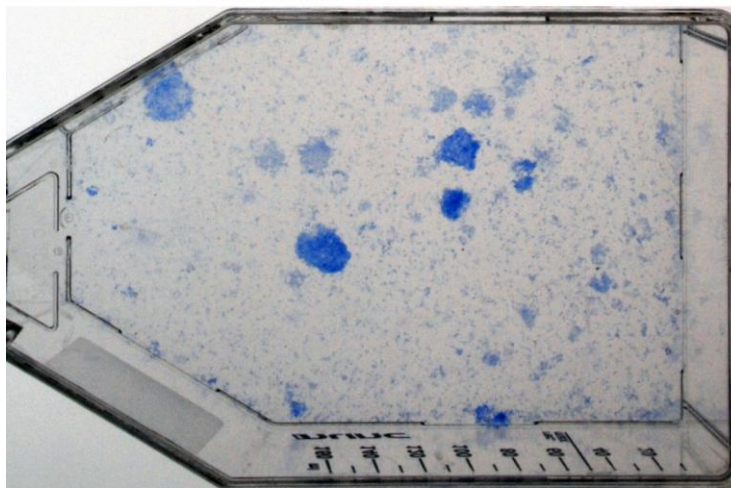


This demonstrated that CCNL1 shRNA 40050 and BRCA1 shRNA 254609 were both sufficient to rescue EcoR Clone 7 cells from the conditional arrest. Because the mix of the other two CCNL1 hairpins also abrogated the growth arrest, it was likely that the abrogation was caused by CCNL1 knock-down rather than off-target effects. Unfortunately the BRCA1 mix was not sufficient to overcome the conditional arrest, so it was unclear if the effect caused by the BRCA1 shRNA 254609 was due to BRCA1 knock-down or off-target effects. The co-transfection with the CCNL1 shRNA 40050 and the BRCA1 shRNA 254609 seemed to achieve slightly better rescue from the conditional arrest than these two shRNAs did separately, but this does not prove that the two genes work together, it could be the compounded effect of silencing two genes which are involved in inducing growth arrest in the EcoR Clone 7 cells through separate actions.

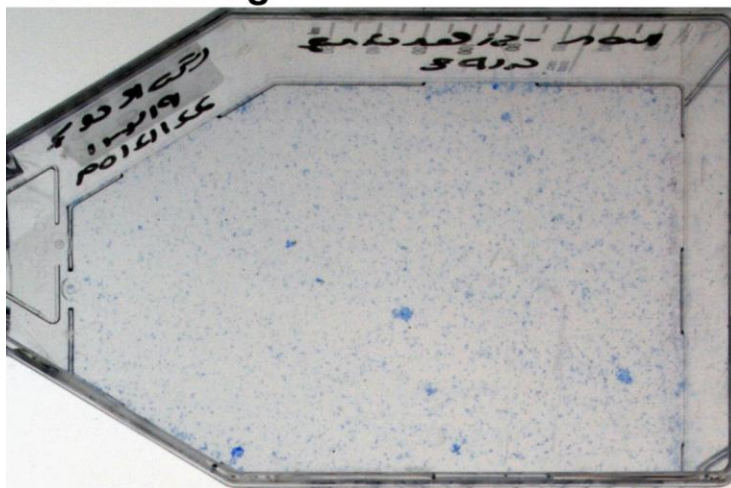
#### **V2LHS\_16814**

The shRNA V2LHS\_16814, which targets FOXA1, was detected in three individual clones in pool 6. V2LHS\_16814 was tested in the EcoR Clone 7 complementation assay. Following incubation at the non-permissive temperature only very low background levels were observed in non-silencing control-infected cells, but many colonies of growing cells were detected in cultures infected with the FOXA1 shRNA. The experiment was set up in triplicate and representative images are shown in Figure 6.12. This demonstrated that the shRNA 16814 was sufficient to overcome the conditional growth arrest.

### FOXA1 shRNA 16814



### Non-silencing



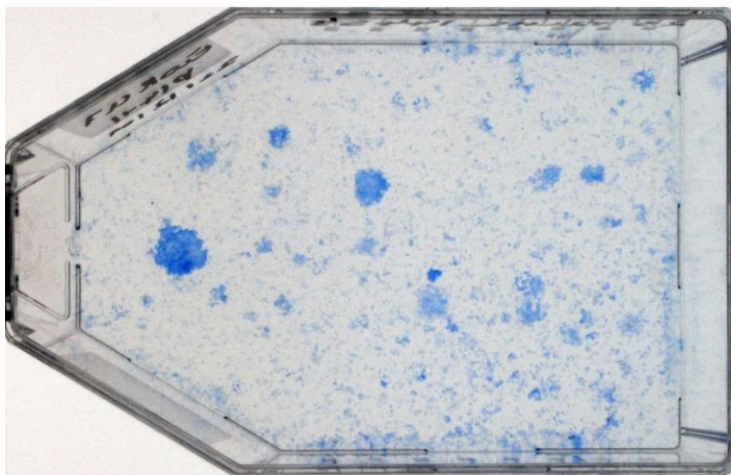
**Figure 6.12: V2LHS\_16814 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The FOXA1 shRNA construct V2LHS\_16814 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 16814, and only very low background levels were detected in cells infected with the non-silencing control virus.

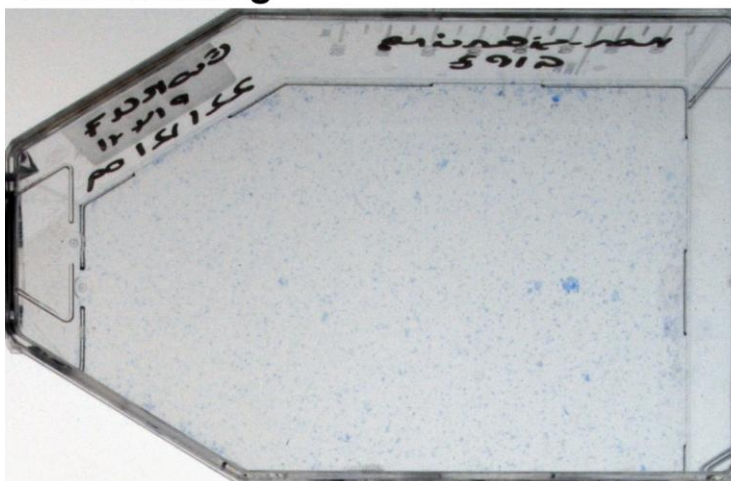
### **V2LHS\_14455**

The SPOPL shRNA V2LHS\_14455, which was detected in pool 7, was tested in the EcoR Clone 7 complementation assay. Following incubation at the non-permissive temperature, very little background growth was detected in the non-silencing control-infected cultures, but multiple colonies of growing cells were detected in cultures infected with the SPOPL shRNA 14455. The experiment was set up in triplicate and representative images are shown in Figure 6.13. This demonstrated that the shRNA 14455 was sufficient to abrogate the conditional growth arrest of EcoR Clone 7 cells.

### SPOPL shRNA 14455



### Non-silencing



**Figure 6.13: V2LHS\_14455 is sufficient to abrogate the EcoR Clone 7 growth arrest**

The SPOPL shRNA construct V2LHS\_14455 was tested in the complementation assay. Colonies of growing cells were visible in cells infected with the hairpin 14455, and only very low background levels were detected in cells infected with the non-silencing control virus.

## 6.5 Discussion

In addition to the loss of function shRNA<sub>mir</sub> screen using the pSM2c library, a loss of function shRNA<sub>mir</sub> screen using the whole genome pGIPZ lentiviral shRNA<sub>mir</sub> library was carried out in the conditional EcoR Clone 7 cells to identify mediators of cellular senescence in human breast epithelial cells. The pSM2c library does not cover the entire genome, only 9,392 genes out of the total number of unique human genes, which is estimated to be 22,500 (International Human Genome Sequencing Consortium, 2004) are represented in this library. Genes which are not covered in this library might be mediators of senescence, for example there are no shRNAs targeting p21 in the pSM2c library, and inhibition of p21 strongly abrogates the conditional growth arrest of EcoR Clone 7 cells. Therefore, it was decided to perform a genome-wide screen utilising the pGIPZ library.

The screen was performed in 7 pools of approximately 9,000 constructs each, and the screen was carried out at an MOI of 0.1 to achieve single inserts as recommended by Open Biosystems. For pool 1 and 2, approximately 2,500,000 cells were infected to achieve 250,000 infectious events, and for pools 3 – 7 approximately 5,000,000 were infected to achieve 500,000 infectious events. It had been calculated that approximately 41,444 infectious events per pool were required to cover the entire library to achieve 99% confidence that each construct in the pool was covered. This amount was increased more than five-fold for pools 1 and 2, and more than ten-fold for pools 3, 4, 5, 6 and 7.

From the 7 pools, a total of 22 candidate hairpins were identified; this is summarised in Table 6.2. Seven of the clones that were picked from the pools, five from pool 7 and two from pool 6, carried multiple inserts and could therefore not be identified directly by sequencing. Such DNAs could have been TOPO-cloned to separate the inserts to enable sequencing, but this was not done due to time restraints. In total, 28 shRNAs were tested in the secondary screen, targeting 22 candidate genes (Table 6.2). Out of these, inhibition of 7 genes led to EcoR Clone 7 cells overcoming the conditional arrest in the secondary screen.

### 6.5.1 pGIPZ library optimisation

Previously it had been demonstrated that the retroviral constructs containing hairpins which target p53 and p21, pRetroSuper-p53 and pRetroSuper-p21, were sufficient to rescue 226L 8/13 and EcoR Clone 7 cells from the conditional growth arrest at the non-permissive temperature (see Chapter 4.2). To test if the pGIPZ library was a compatible system to screen in the EcoR Clone 7 cells, all Expression Arrest pGIPZ constructs targeting p53 (93613, 93615 and 287039) and p21 (230370, 202469, 268120 and 203118) and the non-silencing negative control construct were tested in the EcoR Clone 7 complementation assay (Chapter 3.5.1). The first attempt did not lead to significant rescue, but it indicated that some constructs, specifically 230370, could possibly achieve rescue if the protocol was optimised (Figure 6.2). It is important to mention here that the pRetroSuper-p21 had been selected out of several hairpins by Dr. Mansfield for its strong knock-down efficiency, and pRetroSuper-p53 had also been shown to achieve highly efficient knock-down (Rovillain et al., 2011).

It was then demonstrated that more stringent puromycin selection by increasing the concentration to 6 µg/ml and lowering cell density during selection led to the p21 shRNA 230370 abrogating the growth arrest (Figure 6.4). This was probably achieved by selecting for cells which express the shRNA at a higher level, as it was also shown that increasing puromycin concentration from 2µg to 4µg causes less cells to survive selection, and if concentration is further increased to 6 µg even less cells survive. Cells which are more resistant to puromycin might express the insert better, which would mean that they also express the shRNA better since both puromycin resistance and the shRNA are expressed from a single polycistronic transcript. The finding that more stringent selection led to better rescue, which was believed to be due to better expression of the shRNAs, led to the consideration that using a higher MOI might improve rescue from the growth arrest. Increasing the MOI would mean more inserts per cells, therefore more copies of the shRNA, which would achieve better knock-down (Hannon and Rossi, 2004) of p21/p53 and therefore better abrogation of the growth arrest. It was demonstrated that combining more stringent puromycin selection with infecting EcoR Clone 7 cells at an MOI of 5 with the p21 and p53 pGIPZ constructs led to better abrogation of the growth arrest than infection at an MOI of 0.1 to 0.3. To confirm that this was caused by better knock-down, knock-down of p21 by the four

available shRNAs was tested, and it was shown that knock-down with all constructs was greater at an MOI of 5 than at an MOI of 0.1 to 0.3. Two out of the four p21 shRNAs and one out of the three p53 shRNAs achieved abrogation of the growth arrest even at the low MOI, but significantly less than at the higher MOI. This suggested that, at least in the EcoR Clone 7 cells, the shRNAs in the pGIPZ library did not achieve highly efficient low copy knock-down, which is an important characteristic of an shRNA library to be used for screens. This was in contrast to the claim of Open Biosystems that pGIPZ constructs achieve efficient low copy knockdown making them good for screening in a pooled format.

Infecting cells at an MOI of 5 was a good strategy when testing individual targets, but not for the screen as this strategy relied on improving knock-down by introducing several copies of the same hairpin into a cell, which would be unrealistic in a pooled screen. To perform the screen, single inserts needed to be sufficient to achieve rescue from the conditional arrest. With stringent puromycin selection, abrogation of the growth arrest could be achieved even at an MOI of 0.1 to 0.3 (the MOI recommended by Open Biosystems to achieve single inserts in a pooled screen) with two of the p21 shRNA constructs and one of the p53 shRNA constructs. This indicated that the library was a potentially useful tool to screen for mediators of senescence in EcoR Clone 7 cells. To increase chances of detecting shRNAs which could lead to cells to overcome the growth arrest, the amount of infectious events required for each pool to achieve 99% confidence that each construct in the pool was covered was calculated, and this amount was increased more than five-fold for pools 1 and 2, and more than ten-fold for pools 3, 4, 5, 6 and 7. The titer for the pools was determined at selection with 6 µg puromycin to select for cells that express inserts at slightly higher levels.

### **6.5.2 Sensitivity and saturation**

The effectiveness of the screen was dependent upon its sensitivity and stringency, and in this respect it was important to minimize background levels whilst still detecting all true positive hits. It was important to find a balance between allowing true positives to be detected, whilst minimizing false positives to minimise the workload for the secondary screen. To achieve this, optimal conditions for performing the shRNAmir screen in the EcoR Clone 7 cells had been previously determined by the development of

the EcoR Clone 7 complementation assay (Figure 4.1). The frequency of the event which was being searched for needed to be considered to achieve sufficient sensitivity. The library consisted of 7 pools, each containing approximately 9,000 constructs. Therefore, to test for sensitivity, a 1 in 9,000 mix of the p21 shRNA 230370 positive control construct in non-silencing control construct was packaged and the virus was used to infect 2,500,000 cells at an MOI of 0.1 to achieve 250,000 infectious events. Non-silencing control virus was also used to infect 2,500,000 cells at an MOI of 0.1 to achieve 250,000 infectious events. Following 3 weeks of growth at the non-permissive temperature, 12 colonies were observed in cells infected with the mix, whereas only 3 were observed in non-silencing control infected cells, demonstrating that the complementation assay was sensitive enough to detect constructs in the pool which were able to abrogate the growth arrest. As only one third of the number of colonies (three colonies) were detected in the non-silencing control-infected cultures, it is likely that two thirds of the 12 colonies in cells infected with the mix carried the p21 shRNA. Theoretically, if the p21 shRNA was present at 1 in 9,000, then in 250,000 events it should have been represented 27.7 times, but only 12 outgrowing colonies were observed. This could have been caused by underrepresentation of the p21 shRNA in the viral supernatant, either because it doesn't package as efficiently as the non-silencing control, or because the mix might have been made inaccurately. It is also possible that the MOI was calculated inaccurately and less infectious events were achieved. Another possibility is that not all p21 shRNAs that infected cells caused cells to overcome the growth arrest.

To be 99% confident that the entire library gets screened, i.e. the screen is saturated, the library needed to be searched through approximately 3 times its size, as determined by the following equation:  $\{\ln(1-0.99)/\ln(1-1/(\text{Library Size}))\}$  (<http://www.stanford.edu/group/nolan/screens/screens.html>). The number of infectious events per pool required for a saturated screen was determined to be approximately 41,444. But because the sensitivity experiment discussed above suggested that of an shRNA which can inhibit the growth arrest, only about half of the hairpins introduced into EcoR Clone 7 cells would be detected, the amount of infectious events was increased more than five-fold for pools 1 and 2 (as in the sensitivity experiment), and more than ten-fold for pools 3, 4, 5, 6 and 7 to increase chances of detecting positives.



Even though sensitivity was tested before the screen was performed, and 5 times for pools 1 and 2 and ten times for pools 3-7 the number of infectious events required per pool to achieve a saturated screen was used, the screen was still not fully saturated. This was evident because none of the p21 or p53 shRNA constructs present in the library were detected in the screen. Detection of these constructs would have been an internal control for the screen being saturating. Another indication that the screen was not saturated is the fact that multiple shRNAs targeting the true positive hits CCNL1, FOXA1 and SPOPL were shown to achieve abrogation of the growth arrest (Chapter 7.1.1, Chapter 7.1.2 and Chapter 7.1.4 respectively), but these constructs were not detected in the primary screen.

Not all constructs which can abrogate the EcoR Clone 7 growth arrest being detected in the screen could be due to the fact that only a proportion of cells infected with such constructs overcome the growth arrest (see Figures 6.6, 7.1, 7.3, and 7.8). As for the new targets detected in the screen, they were all tested in the complementation assay using a high MOI and it was never tested if rescue could be achieved had cells been infected at an MOI of 0.1, which had been shown to strongly reduce abrogation of the growth arrest with the p21 and p53 constructs (Figure 6.6). Therefore, these targets might not achieve rescue when used at a low MOI, and might for that reason not have been detected in the screen. With regard to the p21 and p53 constructs which had been shown to achieve abrogation of the conditional arrest (Figure 6.6), it was observed that at an MOI of 0.1 – 0.3 only a proportion of cells overcame the conditional arrest. Therefore, to ensure that the screen was truly saturated, this percentage should have been considered when calculating the amount of infectious events required to achieve a saturated screen. The equation  $\{\ln(1-0.99)/\ln(1-1/(\text{Library Size}))\}$  (<http://www.stanford.edu/group/nolan/screens/screens.html>) which was used to determine the number of infectious events required per pool only achieves 99% confidence that the entire library is screened, but it does not take into consideration that not every time an shRNA is screened leads to abrogation of the growth arrest. If, for example, only one in twenty EcoR Clone 7 cells infected with the p21 shRNA 203118 at an MOI of 0.1 overcomes the growth arrest, then the number obtained from the above formula would need to be multiplied by 20 to obtain the number of infectious events for a saturated screen in regards to this construct. As mentioned above, the amount of

infectious events determined by the formula  $\{\ln(1-0.99)/\ln(1-1/(\text{Library Size}))\}$  was increased more than five-fold for pools 1 and 2 (as in the sensitivity experiment), and more than ten-fold for pools 3, 4, 5, 6 and 7 to increase chances of detecting true positives. But this increase might not have been sufficient, explaining why the screen was not fully saturated. One of the weaker p21 constructs, e.g. 202469, could have been used to determine what proportion of the infected cells (at an MOI of 0.1) overcome the growth arrest, and that value should have been used to determine the amount of infectious events required per pool to achieve a saturated screen.

### **6.5.3 Stringency and false positives**

A total of 30,000,000 cells were infected at an MOI of 0.1 to achieve 3,000,000 infectious events. From these 3,000,000 infectious events a total of 38 clones were picked, 12 of which carried inserts which were shown to overcome the conditional arrest of EcoR Clone 7 cells in a secondary screen (LOC343425 shRNA, FLJ31301 shRNA, LOC351347 shRNA, SPOPL shRNA, BRCA1 shRNA and CCNL1 shRNA in 5 clones, FOXA1 shRNA in 3 clones). This is summarised in Table 6.2. Seven of the clones carried multiple inserts and could therefore not be identified directly by sequencing. Even if these are included in the false positives, then 26 false positives grew from 3,000,000 assayed inserts. That means that 0.00086% of assayed cells continued to grow and produced false positives. This is similar to the rate of false positives that was observed in the pSM2c screen (0.00104%) and also similar to the rate of false positives observed in non-silencing control infected cultures (approximately 1 in 100,000 seeded cells).

### **6.5.4 Alternative to pooled screen**

As discussed above, an MOI aiming for single inserts does not lead to very efficient knock-down and abrogation of the growth arrest even with the p21 and p53 pGIPZ constructs. Furthermore, the targets detected in the screen only abrogated the growth arrest in a proportion of the cells infected even at a high MOI, and would most likely be less efficient at an MOI which achieves single inserts. To detect shRNAs which can abrogate the conditional arrest in a pooled screen, it is important that single inserts can be detected. To allow the use of a higher MOI, performing the screen in a 96-well format, testing one shRNA per well or a pool of all shRNAs designed to target the same

gene per well might be more efficient than performing the screen in a pooled format. In such a set-up, a high MOI could have been used to achieve multiple shRNAs targeting the same gene per cells, to achieve maximum knock-down and therefore improve chances of detecting true positives. Plates could have been stained for BrdU and analysed with an InCell 1000 automated microscope to detect constructs which cause a higher percentage of BrdU positive cells than the non-silencing control [as used by Borgdorff and colleagues (Borgdorff et al., 2010)]. Due to the large scale of this approach it might not be feasible, and it would not be feasible to perform such a screen without robotics: To screen 62,000 constructs in 96-well plates with only one well per construct, 646 plates would be required. It might not be possible to infect cells in the 96-well plates and directly screen for abrogation of the growth arrest because of the need of puromycin selection and the importance of cell density in the complementation assay, making this approach even more labour intensive and complex. Finally, it would not be feasible to grow bacteria to harvest DNA and then produce virus from 62,000 constructs, but it would be too expensive to purchase the individually packaged high titer viruses available from Open Biosystems. It might be more feasible to follow the approach used by Borgdorff and colleagues (Borgdorff et al., 2010), who used the Silencer whole genome synthetic siRNA library from Ambion (Austin, TX, USA). This library consists of 22,010 siRNA pools (based on NCBI's RefSeq database release 12) with three siRNAs per pool.

### **6.5.5 Secondary screen**

#### **POOL 5**

The CCNL1 shRNA 40050 and the BRCA1 shRNA 254609 were detected together in five individually picked clones in pool 5. Because this pool had been reseeded, the five clones most likely stemmed from the same infectious event. These two constructs were tested together and separately. It seemed like rescue was improved by co-infection, but this does not mean that they worked together to achieve better abrogation of the growth arrest. They might have separate effects, but by affecting two separate pathways involved in the induction of senescence a stronger abrogation of the growth arrest could be achieved. Both hairpins caused abrogation of the growth arrest when introduced into

EcoR Clone 7 cells separately, so the effect is not dependent on knock-down of both genes. This would need to be further investigated to expose if BRCA1 and CCNL1 cooperate in inducing cellular senescence in EcoR Clone 7 cells.

#### **6.5.6 Conclusion**

In conclusion, 7 shRNAs which abrogate the EcoR Clone 7 conditional arrest were identified with the pGIPZ library screen. To determine if abrogation of the growth arrest was caused by knock-down of the genes which these hairpins were designed to knock down was still unclear, to do so off-target effects needed to be ruled out and knock-down of target genes needed to be demonstrated. This was further investigated and will be discussed in Chapter 7.

## **7 Results – Further validation of target genes**

12 shRNAs identified in the pSM2c and pGIPZ screen had been validated in a secondary screen. To rule out off-target effects, several hairpins targeting the same gene had to be tested in the EcoR Clone 7 complementation assay. As before, EcoR Clone 7 cells frozen in bulk at passage 12/13 were used for the validation screen. The complementation assay was used as described in section 3.5.1, with three modifications: Puromycin selection was started 24 hours instead of 48 hours post-infection at 6µg/ml instead of 2µg/ml. Also, cells were infected at a high MOI (5 ml of virus per 80,000 cells). pGIPZ non-silencing control (scrambled) was used as a negative control, and the p21 shRNA V2LHS\_268120 as the positive control.

Furthermore, knock-down of target genes achieved by the shRNAs was analysed, on the RNA level, and where possible also on the protein level.

### **7.1 Known genes**

Several hairpins targeting known genes were identified in the screen and confirmed as true positives in a secondary screen. When hairpins overcame the conditional arrest in a secondary screen, several hairpins targeting the same gene were ordered and assayed for ability to rescue EcoR Clone 7 cells from the conditional growth arrest. This was important to show that rescue was not caused by off-target effects, meaning knock-down of genes other than the one the hairpins were designed against.

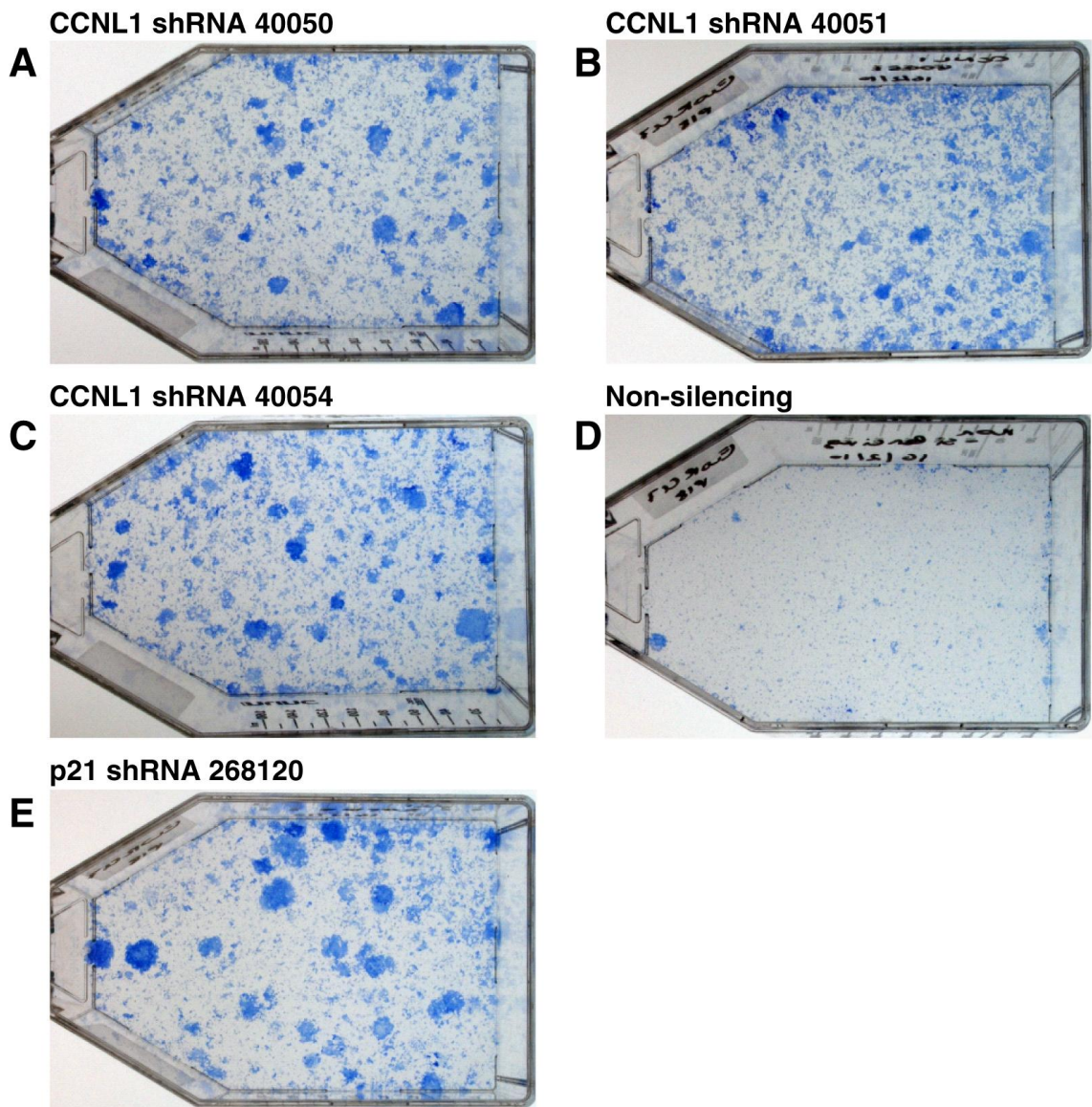
In addition to showing that multiple hairpins achieved rescue, knock-down was demonstrated with real-time quantitative RT-PCR and where antibodies were available also by western blotting. When cells were reseeded for the growth assay, cultures were set up at 34°C and 38°C for harvesting protein and RNA. For protein extractions, at the permissive temperature T75 flasks were grown to approximately 80% confluence and cells were lysed with RIPA buffer. At the non-permissive temperature, non-silencing control-infected cultures were pooled 3 days before protein was harvested to achieve 80% confluence as these cultures don't grow. Protein concentration was determined using the Bradford assay. To show knock-down at the RNA level, real-time quantitative RT-PCR was performed using the Ambion Cells-to-CT kit and gene specific TaqMan

Gene Expression Assays (containing primer sets and probes). This was performed on cells grown at 34°C for CCNL1, Rab23 and FOXA1, and on cells incubated at 38°C for SPOPL. Cells infected with the non-silencing control and the shRNA constructs were seeded at 10,000 cells in a 96 well plate. The following day cells were lysed in the presence of DNase, which was followed by an RT reaction. cDNA products were then used for the real-time PCR reaction.

### **7.1.1 CCNL1**

#### ***7.1.1.1 Off Target Effects***

The CCNL1 shRNA 40050 and a mix of the hairpins 40051 and 40054 had been shown to enable EcoR Clone 7 cells to overcome the conditional arrest. To rule out off-target effects, the three CCNL1 shRNAs were tested separately in the EcoR Clone 7 growth assay. The non-silencing control and p21 shRNA 268120 were used as the negative and positive control respectively. The experiment was done in triplicate, and representative images are shown in Figure 7.1. Each time many growing clones were detected in all CCNL1 infected cultures, but only very few were detected in non-silencing control-infected cultures. This result indicated that CCNL1 knock-down was sufficient to overcome the EcoR Clone 7 temperature dependent growth arrest.



**Figure 7.1: Several CCNL1 shRNAs abrogate the EcoR Clone 7 growth arrest**

The CCNL1 shRNAs 40050, 40051 and 40054 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (A) 40050, (B) 40051 and (C) 40054. (D) Only very low background levels were detected in cells infected with the non-silencing control virus. (E) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

### **7.1.1.2 Knock-down**

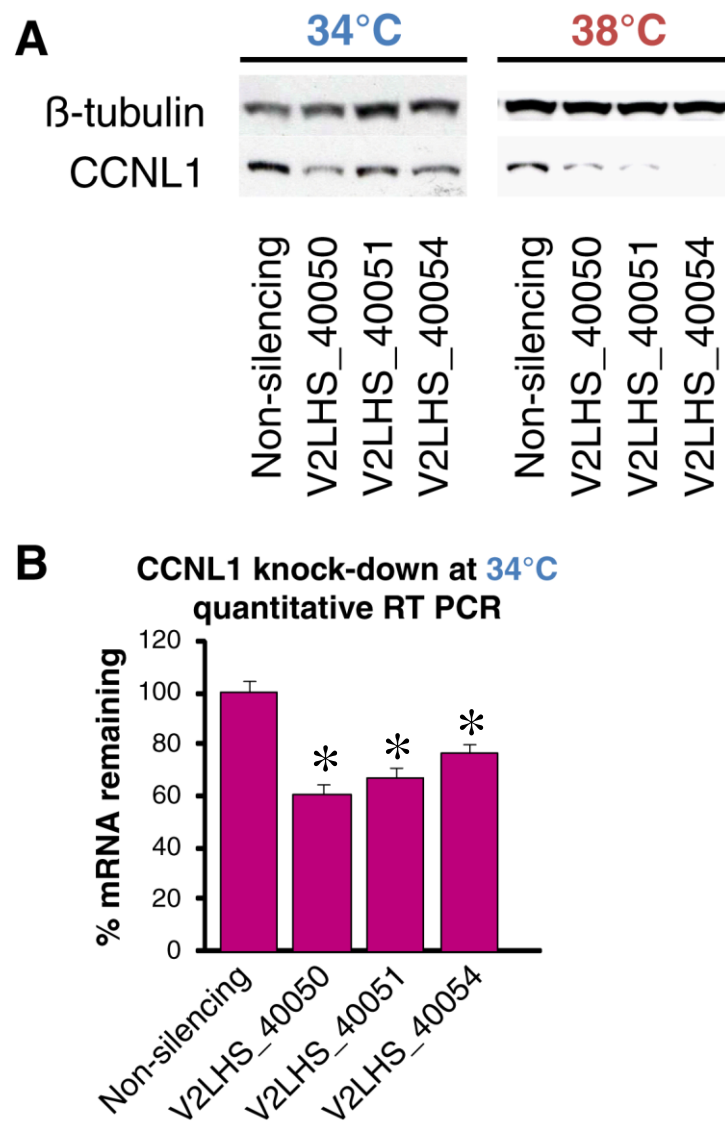
#### **7.1.1.2.1 Western Blotting**

70 µg of protein were loaded on a 10% gel with Kaleidoscope protein size marker. CCNL1 antibody (a kind gift from Dr. Lahti) was used at a dilution of 1 in 1000 in 5% milk PBS Tween for 1 hour at room temperature. Secondary anti-rabbit antibody was used at 1 in 10,000 in 5% milk PBS Tween for one hour at room temperature.  $\beta$ -tubulin was used as a control for equal protein loading. Figure 7.2 A shows the knock-down in cells grown at 34°C and at 38°C. Compared to the non-silencing control, the approximately 64kDa band corresponding to CCNL1 [as indicated in the literature (Loyer et al., 2008)] was strongly reduced by all 3 shRNAs. Interestingly, at 34°C the shRNA 40050 seemed to produce the strongest knock-down, whereas at 38°C the band was reduced the most by the shRNA 40054. The western blot demonstrated that all three CCNL1 shRNAs reduced CCNL1 protein levels, and knock-down in cells grown at 38°C was greater than in cells grown at 34°C, suggesting that cells with better knock-down were selected for in the growth assay.

#### **7.1.1.2.2 Real-time quantitative RT-PCR**

To show knock-down at the RNA level, real-time quantitative RT-PCR was performed using the Ambion Cells-to-CT kit and a CCNL1 TaqMan Gene Expression Assay. Figure 7.2 B shows the percentage of mRNA remaining in cells infected with the three CCNL1 shRNAs as compared to the total mRNA level in non-silencing control infected cells, assumed to be 100%. The shRNA 40050 reduced mRNA levels to 61% of total mRNA remaining, 40051 to 66.5%, and 40054 to 76.6%. To summarise, all three shRNAs reduced CCNL1 mRNA levels.





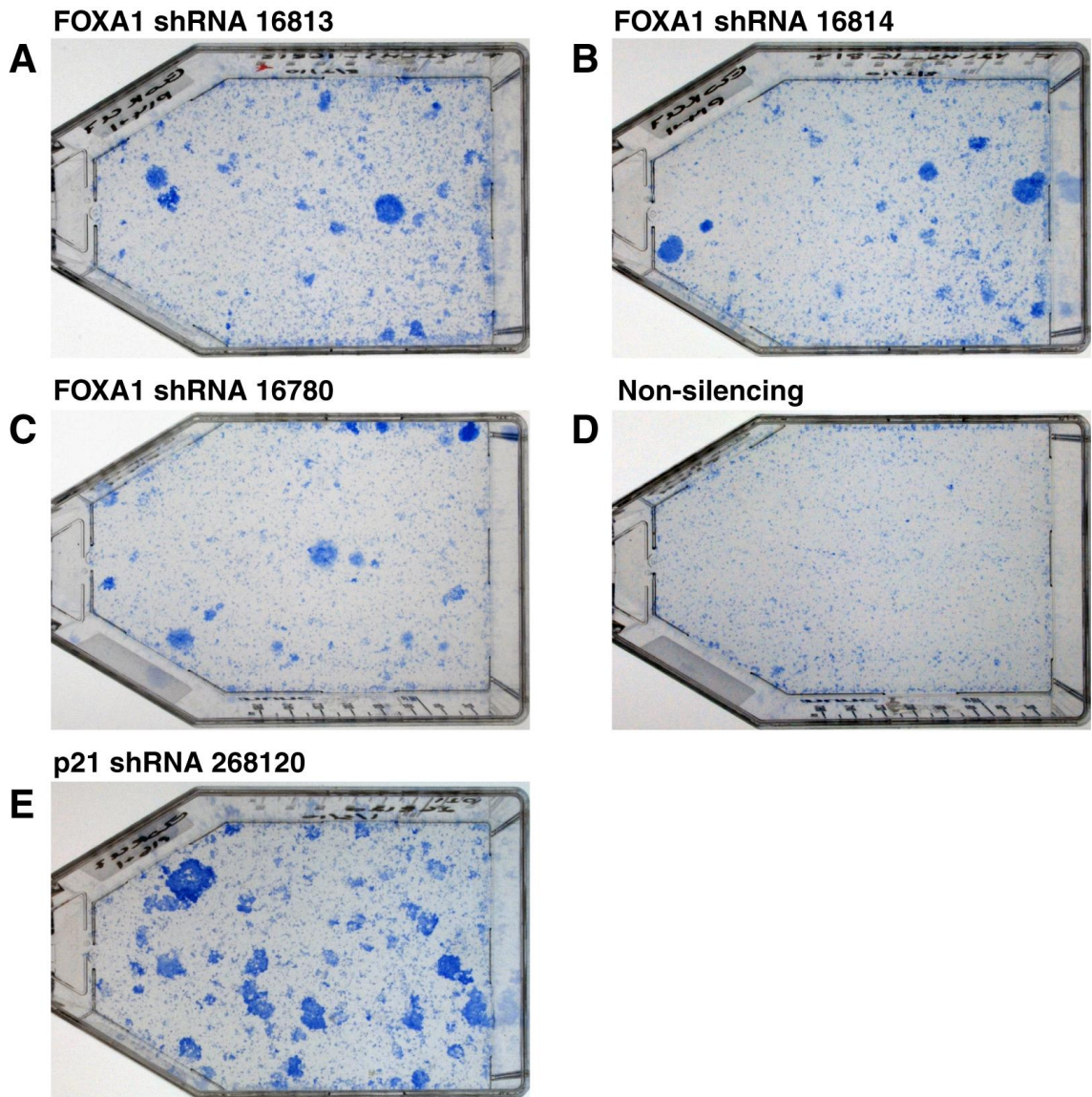
**Figure 7.2: Knock-down achieved by the CCNL1 shRNAs**

(A) Western blotting demonstrated CCNL1 silencing at 34°C and at 38°C. β-tubulin was used as the loading control. (B) Quantitative RT-PCR demonstrated CCNL1 silencing at 34°C. Bars represent normalised expression values relative to β-Actin. Error bars represent the standard error of the mean. \* indicates  $p \leq 0.05$  versus non-silencing control, Student's t-test, one-tailed distribution assuming equal variance.

### **7.1.2 FOXA1**

#### ***7.1.2.1 Off Target Effects***

The FOXA1 shRNA 16814 was found in multiple clones in the screen, and it was confirmed to work in the secondary screen. Three hairpins targeting this gene were available from the Expression Arrest pGIPZ library, V2LHS\_16814, V2LHS\_16813 and V2LHS\_16780. These shRNAs were tested for their ability to rescue EcoR Clone 7 cells from the conditional growth arrest. Three replicates were set up for each shRNA. Following incubation at 38°C for two weeks, cultures were fixed and stained with methylene blue; representative flasks are shown in Figure 7.3. Very low levels of background growth were detected in non-silencing control-infected cultures, but multiple colonies of growing cells were detected in cultures infected with the FOXA1 shRNAs. The constructs 16813 and 16814 were equally efficient in overcoming the growth arrest, and the construct 16780 was slightly less efficient. Abrogation of the growth arrest by the FOXA1 shRNAs was not as great as that achieved by knocking down p21 using the construct 268120. This result indicated that FOXA1 knock-down was sufficient to overcome the EcoR Clone 7 conditional growth arrest.



**Figure 7.3: Several FOXA1 shRNAs abrogate the EcoR Clone 7 growth arrest**

The FOXA1 shRNAs 16813, 16814 and 16780 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (**A**) 16813, (**B**) 16814 and (**C**) 16780. (**D**) Only very low background levels were detected in cells infected with the non-silencing control virus. (**E**) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

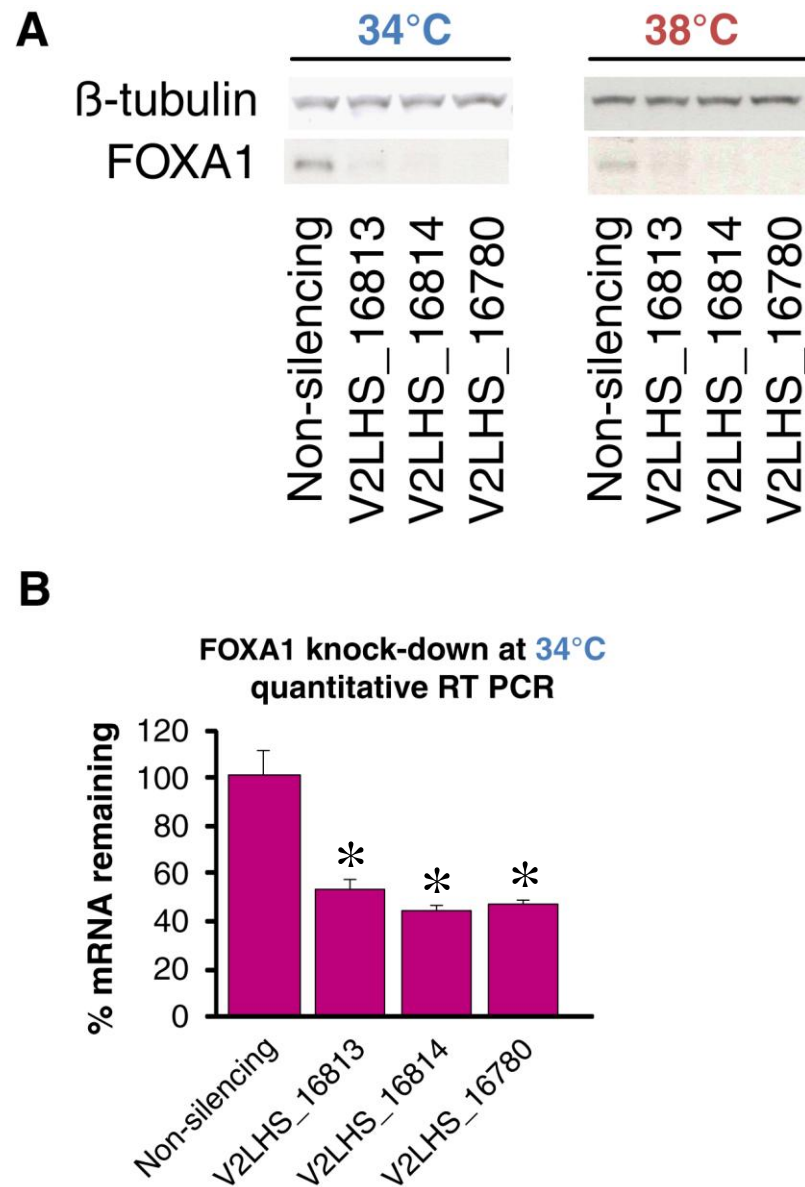
### **7.1.2.2 Knock-down**

#### **7.1.2.2.1 Western Blotting**

35 µg of protein were loaded on a 10% gel with Kaleidoscope protein size marker. FOXA1 antibody was used at a dilution of 1 in 500 in 5% milk PBS Tween and incubated at 4°C over night. Secondary anti-rabbit antibody was used at 1 in 10,000 in 5% milk PBS Tween for one hour at room temperature. β-tubulin was used as a control for equal protein loading. Figure 7.4 A shows the knock-down in cells grown at 34°C and in cells grown at 38°C. Only one band was visible at the size corresponding to FOXA1, and compared to the non-silencing control, the approximately 49kDa band corresponding to FOXA1 was strongly reduced by all 3 constructs at both temperatures.

#### **7.1.2.2.2 Real-time quantitative RT-PCR**

To show knock-down at the RNA level, real-time quantitative RT-PCR was performed using the Ambion Cells-to-CT kit and a FOXA1 TaqMan Gene Expression Assay. Figure 7.4 B shows the percentage of mRNA remaining in cells infected with the three FOXA1 shRNAs as compared to the total mRNA level (100%) in non-silencing control-infected cells. The shRNA 16813 reduced mRNA levels to 47.5%, 16814 to 54%, and 16780 to 45%. In summary, all three FOXA1 shRNAs reduced mRNA levels to approximately 50%. Generally, knock-down of protein levels observed by western blotting appeared greater, which might be due to the shRNAs ability to not only degrade mRNA levels, but also inhibit translation of mRNA. This might also be due to the western blotting being less sensitive than the q PCR.



**Figure 7.4: Knock-down achieved by the FOXA1 shRNAs**

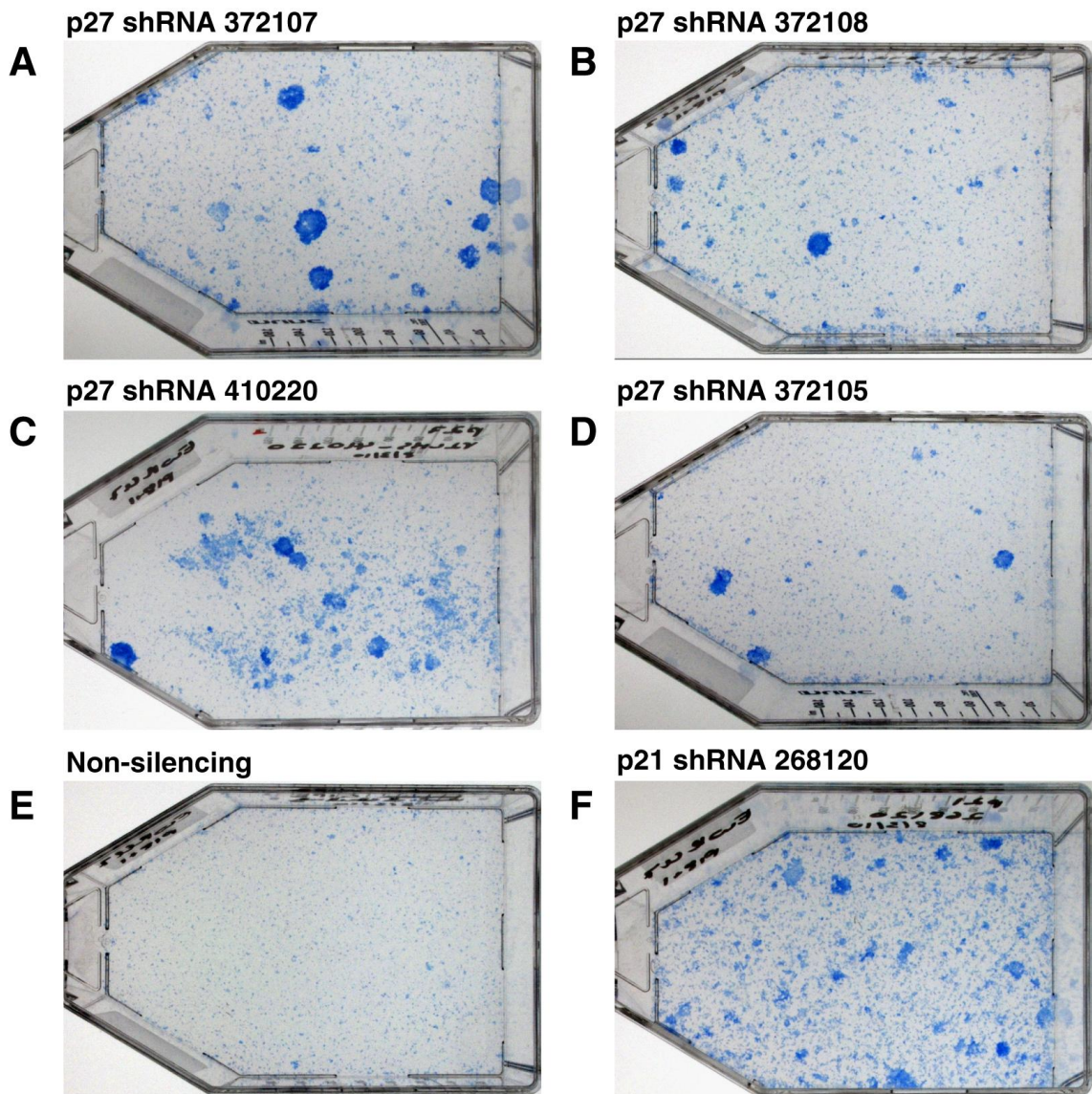
(A) Western blotting demonstrated FOXA1 silencing at 34°C and at 38°C.  $\beta$ -tubulin was used as the loading control. (B) Quantitative RT-PCR demonstrated FOXA1 silencing at 34°C. Bars represent normalised expression values relative to  $\beta$ -Actin. Error bars represent the standard error of the mean. \* indicates  $p \leq 0.05$  versus non-silencing control, Student's t-test, one-tailed distribution assuming equal variance.

### ***7.1.2.3 Knock-down of p27<sup>Kip1</sup> is sufficient to rescue EcoR Clone 7 cells from the conditional growth arrest***

As discussed above, FOXA1 can activate the promoter of p27. P27 is a member of the Cip/Kip family of CDK inhibitors, which includes p21/WAF/CIP1 and p57/kip2 (Eldeiry et al., 1993; Lee et al., 1995; Matsuoka et al., 1995; Toyoshima and Hunter, 1994; Xiong et al., 1993). p27 expression is regulated by cell contact inhibition and by specific growth factors, such as transforming growth factor (TGF)- $\beta$  (Polyak et al., 1994a), and it suppresses the activity of cyclin E-CDK2 (Blain et al., 1997; LaBaer et al., 1997; Soos et al., 1996).

As FOXA1 can activate the p27 promoter it was not unreasonable to postulate that it exerts a growth inhibiting effect in the EcoR Clone 7 cells via activation of p27 transcription. Therefore, the hypothesis that knock-down of p27 could cause EcoR Clone 7 cells to overcome the conditional arrest was tested. Four pGIPZ vectors targeting p27, namely V2LHS\_372108, V2LHS\_372107, V2LHS\_410220, and V2LHS\_372105 were tested for their ability to overcome the conditional arrest of EcoR Clone 7 cells in the complementation assay. Non-silencing control was used as the negative, and p21 V2LHS\_268120 as the positive control. The experiment was set up in triplicate, and representative flasks are shown in Figure 7.5. Very low background levels were observed in non-silencing control infected cells. In cultures infected with the p27 shRNAs 372108, 372107, and 410220 growing colonies were detected. The number of growing colonies in these cultures was not as high as in p21 knock-down cultures infected with the hairpin 268120. In cultures infected with one of the constructs tested, 372105, very few growing colonies were visible. These results indicate that inhibition of p27 is sufficient to abrogate the EcoR Clone 7 growth arrest. This is consistent with the hypothesis that FOXA1 induces senescence arrest by activating p27 expression in the EcoR Clone 7 cells.





**Figure 7.5: Several p27 shRNAs abrogate the EcoR Clone 7 growth arrest**

The p27 shRNAs 372107, 372108, 410220 and 372105 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (A) 372107, (B) 372108 (C) 410220 and (D) 372105. (E) Only very low background levels were detected in cells infected with the non-silencing control virus. (F) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

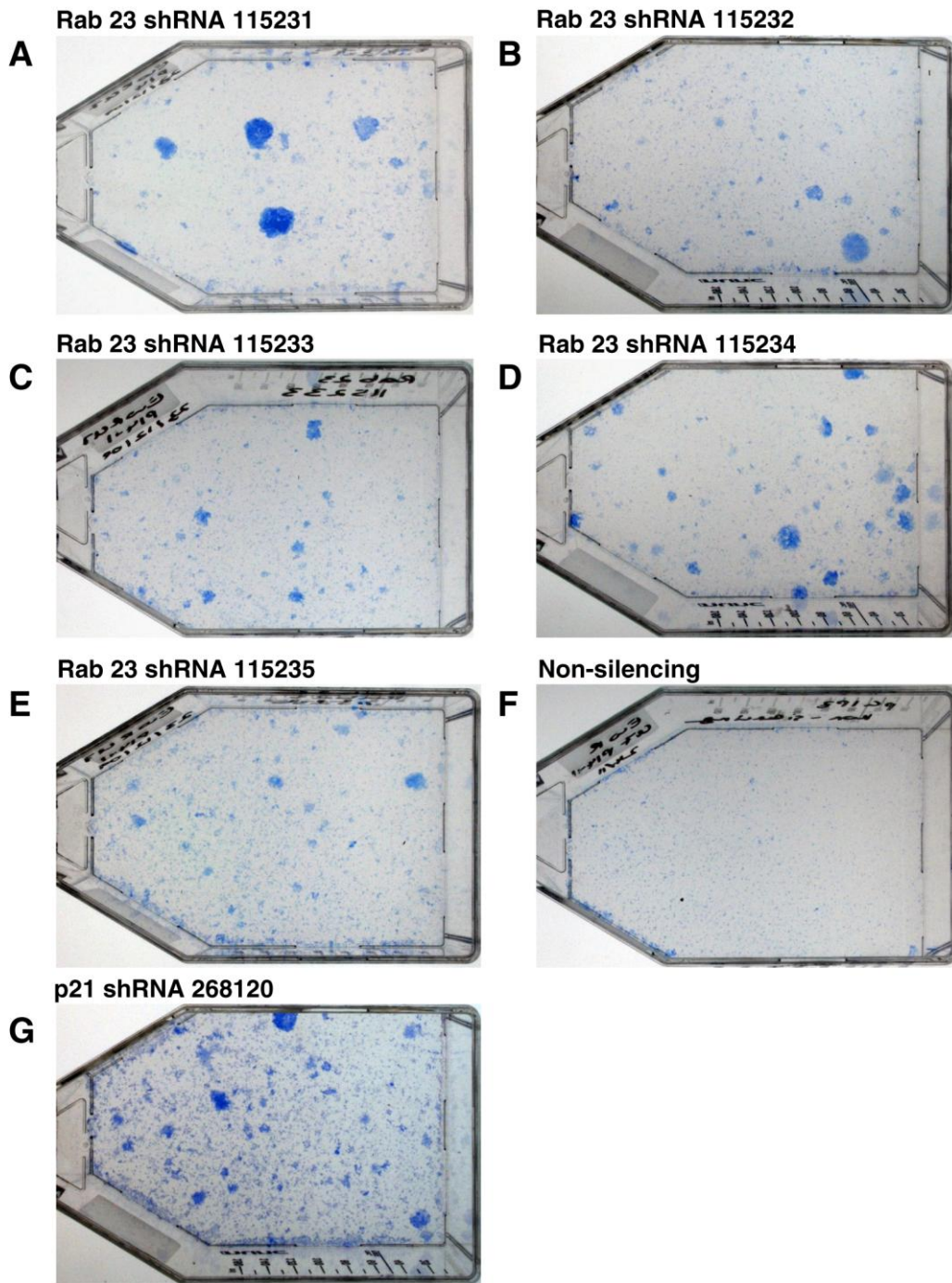
### **7.1.3 Rab23**

#### ***7.1.3.1 Off Target Effects***

The Rab23 shRNA 115231 was detected in two clones in pool 56 which had not been reseeded, and it was confirmed to work in a secondary screen.

Four additional hairpins targeting this gene which were available from the Expression Arrest pGIPZ library, V2LHS\_115232, V2LHS\_115233, V2LHS\_115234, and V2LHS\_115235, were tested for their ability to overcome the EcoR Clone 7 conditional growth arrest. Three infections were set up for each construct, and representative images are shown in Figure 7.6. Very low background levels were detected in non-silencing control-infected cultures. Multiple colonies of growing cells were detected in cultures infected with the Rab23 shRNA constructs. Generally rescue was not as great as that achieved by knocking down p21 using the construct 268120. This result demonstrated that Rab23 knock-down was sufficient to overcome the EcoR Clone 7 conditional growth arrest.





**Figure 7.6: Several Rab23 shRNAs abrogate the EcoR Clone 7 growth arrest**

The Rab23 shRNAs 115231, 115232, 115233, 115234, and 115235 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (A) 115231, (B) 115232 (C) 115233 (D) 115234 and (E) 372105. (F) Only very low background levels were detected in cells infected with the non-silencing control virus. (G) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

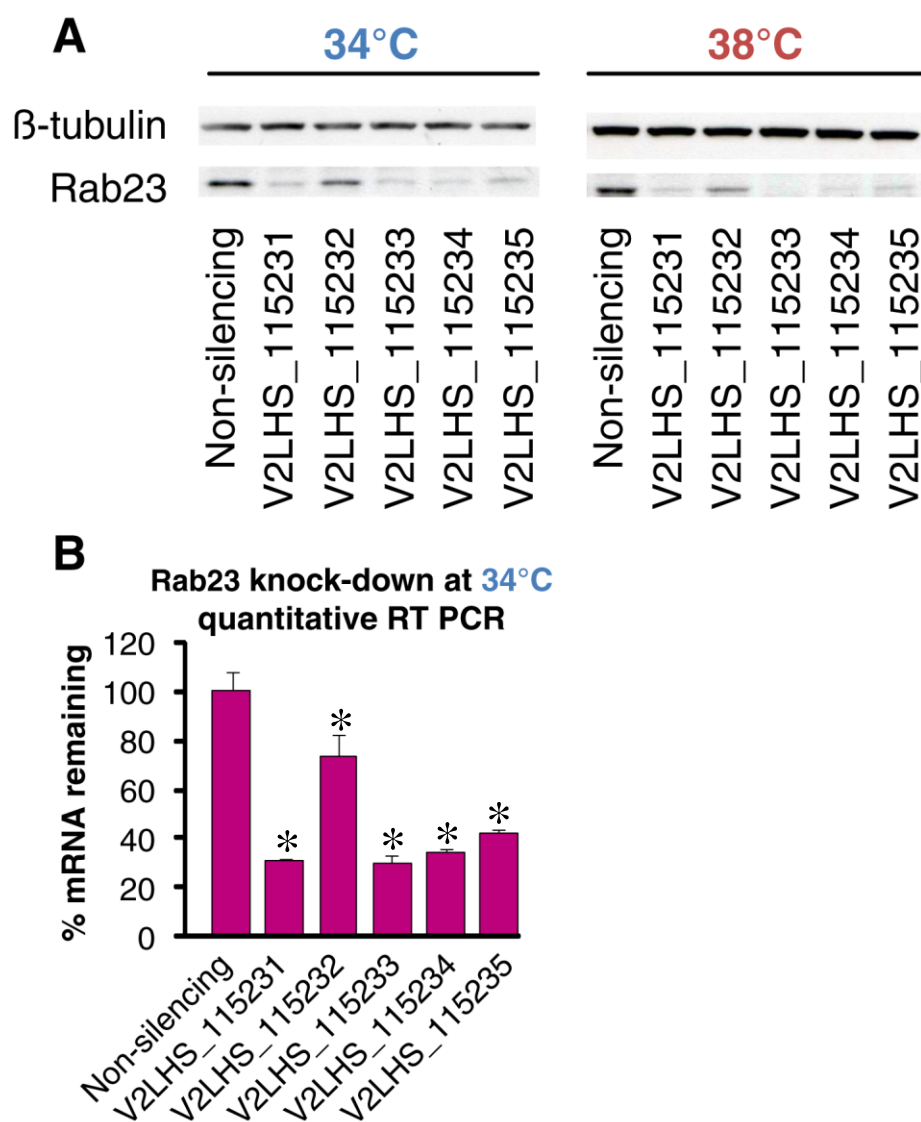
### **7.1.3.2 Knock-down**

#### **7.1.3.2.1 Western Blotting**

35 µg of protein were loaded on a 16% gel with Kaleidoscope protein size marker. Rab23 antibody (a kind gift from Professor Bor Luen Tang) was used at a dilution of 1 in 500 in 3% BSA PBS Tween and incubated at 4°C over night. Secondary anti-rabbit antibody was used at 1 in 10,000 in 5% milk PBS Tween for one hour at room temperature.  $\beta$ -tubulin was used as a control for equal protein loading. Figure 7.7 A shows Rab23 knock-down by the shRNA constructs in cells grown at 34°C and cells grown at 38°C. At both temperatures the approximately 30kDa band corresponding to Rab23 was strongly reduced by the 4 of the shRNAs compared to the band seen in the non-silencing control, namely 115231 (detected in the screen), 115233 and 115234 and 115235. The hairpin 115232 was the least efficient at reducing the band, which correlated with it having the weakest effect in the complementation assay. In Summary, efficient silencing of Rab23 protein levels was achieved by four of the shRNAs, namely 115321, 115233, 115234 and 115235, and slightly less knock-down by 115232.

#### **7.1.3.2.2 Real-time quantitative RT-PCR**

RT-PCR was performed using the Ambion Cells-to-CT kit and a Rab23 TaqMan Gene Expression Assay. Figure 7.7 B shows the percentage of mRNA remaining in cells infected with the five Rab23 shRNAs as compared to the total mRNA level (100%) in non-silencing control-infected cells. The shRNA 115231 reduced mRNA levels to 30.6%, 115233 to 30.4% and 115234 to 34.2%. The shRNA 115235 reduced mRNA levels slightly less efficiently, with the level remaining at 42.1%, and the hairpin 115232 only achieved a reduction to 73.2%. The knock-down of mRNA by the specific shRNAs correlated to the reduction of protein levels by the shRNAs observed by western blotting, with 115321, 115233, 115234 being the most efficient, followed by 115325, and the least efficient shRNA being 115232. Generally, knock-down of protein levels appeared more efficient than knock-down of mRNA, which might be due to the shRNAs ability to not only cause degradation of the mRNA, but to also inhibit its translation, or due to the western blotting being less sensitive.



**Figure 7.7: Knock-down achieved by the Rab23 shRNAs**

(A) Western blotting demonstrated Rab23 silencing at 34°C and at 38°C.  $\beta$ -tubulin was used as the loading control. (B) Quantitative RT-PCR demonstrated Rab23 silencing at 34°C. Bars represent normalised expression values relative to  $\beta$ -Actin. Error bars represent the standard error of the mean. \* indicates  $p \leq 0.05$  versus non-silencing control, Student's t-test, one-tailed distribution assuming equal variance.

#### **7.1.4 SPOPL**

##### ***7.1.4.1 Off Target Effects***

The SPOPL shRNA V2LHS\_14455 was detected in pool 7 of the Expression Arrest pGIPZ screen, and it was confirmed to work in the secondary screen. Two additional hairpins targeting this gene were available from the Expression Arrest pGIPZ library, V2LHS\_165322 and V2LHS\_254535, and they were tested for their ability to abrogate the EcoR Clone 7 conditional growth arrest in the complementation assay.

The experiment was set up in triplicate, and representative flasks are shown in Figure 7.8 A-E. Very low background levels were detected in non-silencing control-infected cultures. In cultures infected with the three hairpins targeting SPOPL colonies of growing cells were detected, and abrogation of the growth arrest appeared most efficient with the hairpin 14455 which had been isolated in the screen. None of the constructs abrogated the growth arrest as efficiently as the p21 shRNA 268120. These results indicate that knock-down of SPOPL can lead to EcoR Clone 7 cells overcoming the conditional arrest at the non-permissive temperature.

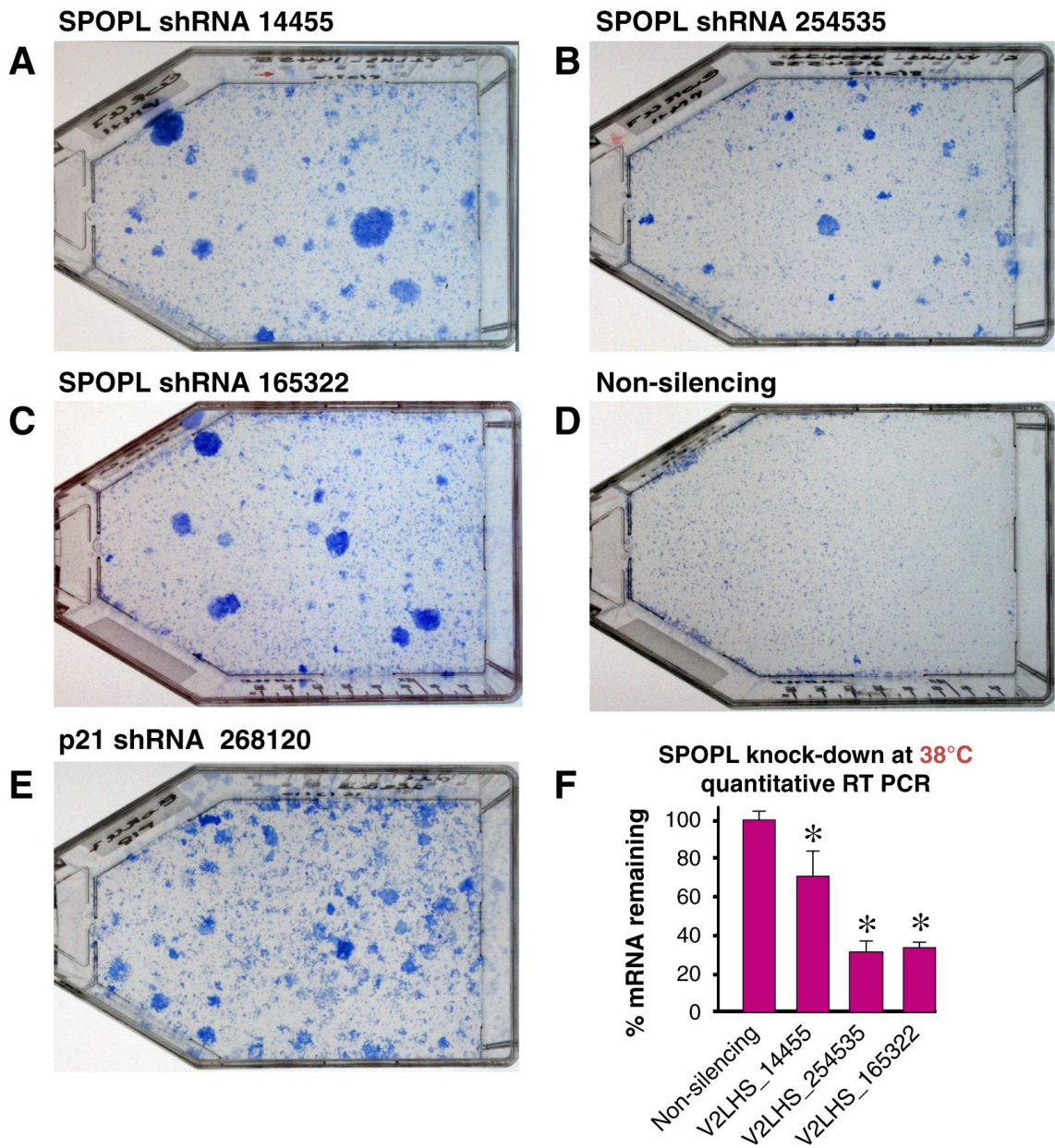
##### ***7.1.4.2 Knock-down***

To show that the shRNAs knocked down SPOPL, real-time quantitative RT-PCR was performed. No suitable antibody was found; therefore western blotting to show knock-down of SPOPL at the protein level could not be done.

###### ***7.1.4.2.1 Real-time quantitative RT-PCR***

RT-PCR was performed using the Ambion Cells-to-CT kit and an SPOPL TaqMan Gene Expression Assay. Figure 7.8 F shows the percentage of mRNA remaining in cells infected with the three SPOPL shRNAs as compared to the total mRNA level (100%) in non-silencing control-infected cells. The shRNA 14455 reduced mRNA levels to 70.7%, 254535 knocked levels down to 31.8% and 165322 to 33.3% of the total mRNA. It was surprising that the construct 14455 which had been detected in the screen and also abrogated the growth arrest most efficiently caused the least significant reduction in mRNA levels.





**Figure 7.8: Several SPOPL shRNAs abrogate the EcoR Clone 7 growth arrest**

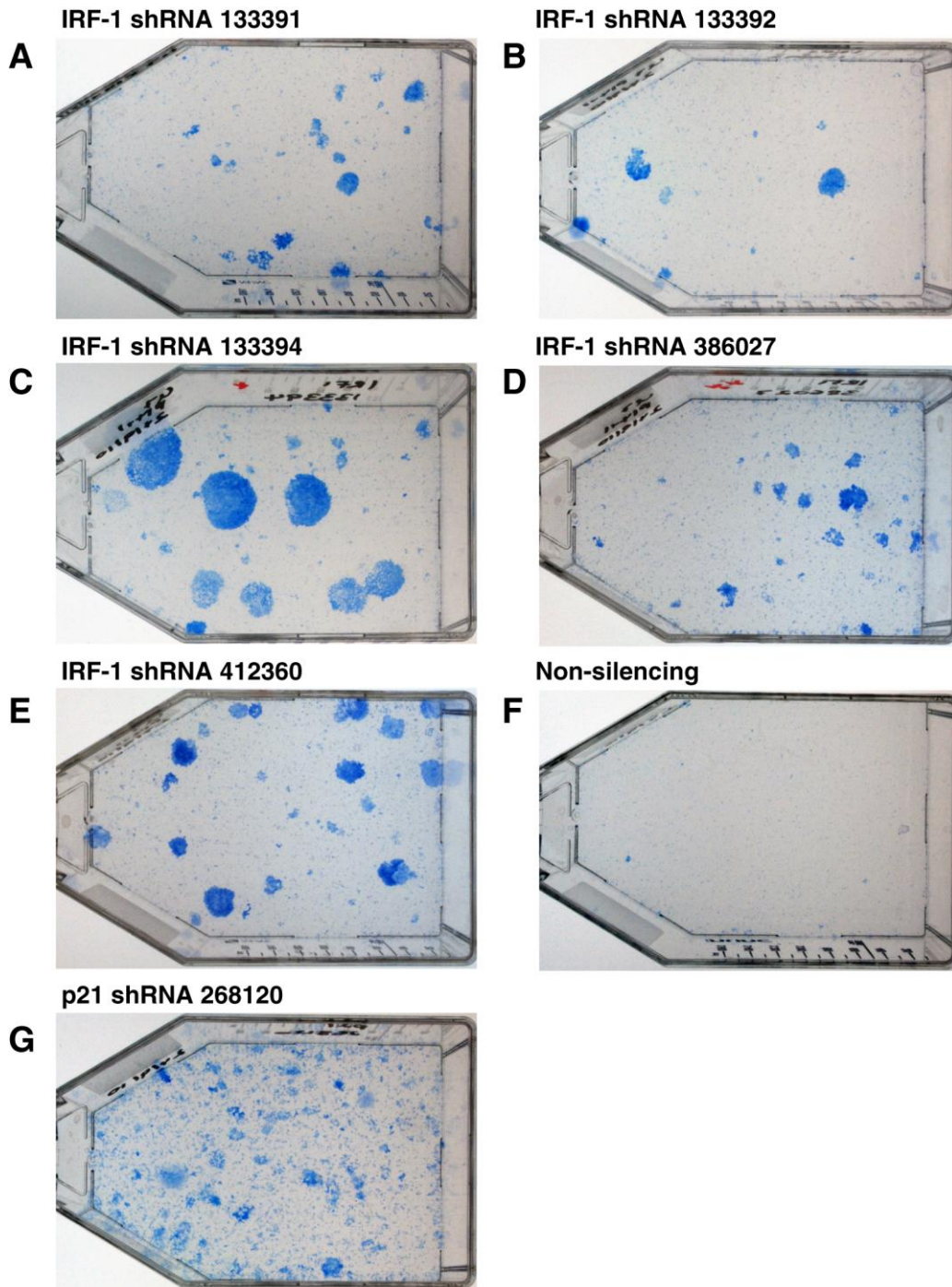
The SPOPL shRNAs 14455, 165322 and 254535 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (A) 14455, (B) 254535 and (C) 165322. (D) Only very low background levels were detected in cells infected with the non-silencing control virus. (E) Abrogation of the growth arrest achieved by the p21 shRNA 268120. (F) Quantitative RT-PCR demonstrated SPOPL silencing at 38°C. Bars represent normalised expression values relative to  $\beta$ -Actin. Error bars represent the standard error of the mean. \* indicates  $p \leq 0.05$  versus non-silencing control, Student's t-test, one-tailed distribution assuming equal variance.

### 7.1.5 IRF-1

#### 7.1.5.1 *Off-target effects*

The IRF-1 shRNA 133394 was detected in pool 96 of the retroviral pSM2c screen, and when it was tested in a secondary screen using the pGIPZ construct V2LHS\_133394 it led to EcoR Clone 7 cells overcoming the conditional arrest.

Four additional hairpins targeting IRF-1 were available from the Expression Arrest pGIPZ library, V2LHS\_133391, V2LHS\_133392, V3LHS\_386027, and V3LHS\_412360. All five hairpins were tested in the EcoR Clone 7 complementation assay. The experiment was set up in triplicate. Following growth at the permissive temperature for 2.5 weeks, cultures were fixed and stained with methylene blue, and representative images are shown in Figure 7.9. Very low background levels were detected in non-silencing control-infected cultures. The shRNA 133394, which had been isolated in the screen, abrogated the growth arrest most efficiently. Four of the IRF-1 shRNAs, namely 133391, 133394, 386027 and 412360 produced multiple healthy looking colonies of growing cells. In cultures infected with the shRNA 133392 less colonies were observed. This result demonstrated that IRF-1 knock-down was sufficient to abrogate the EcoR Clone 7 conditional growth arrest.



**Figure 7.9: Several IRF-1 shRNAs abrogate the EcoR Clone 7 growth arrest**

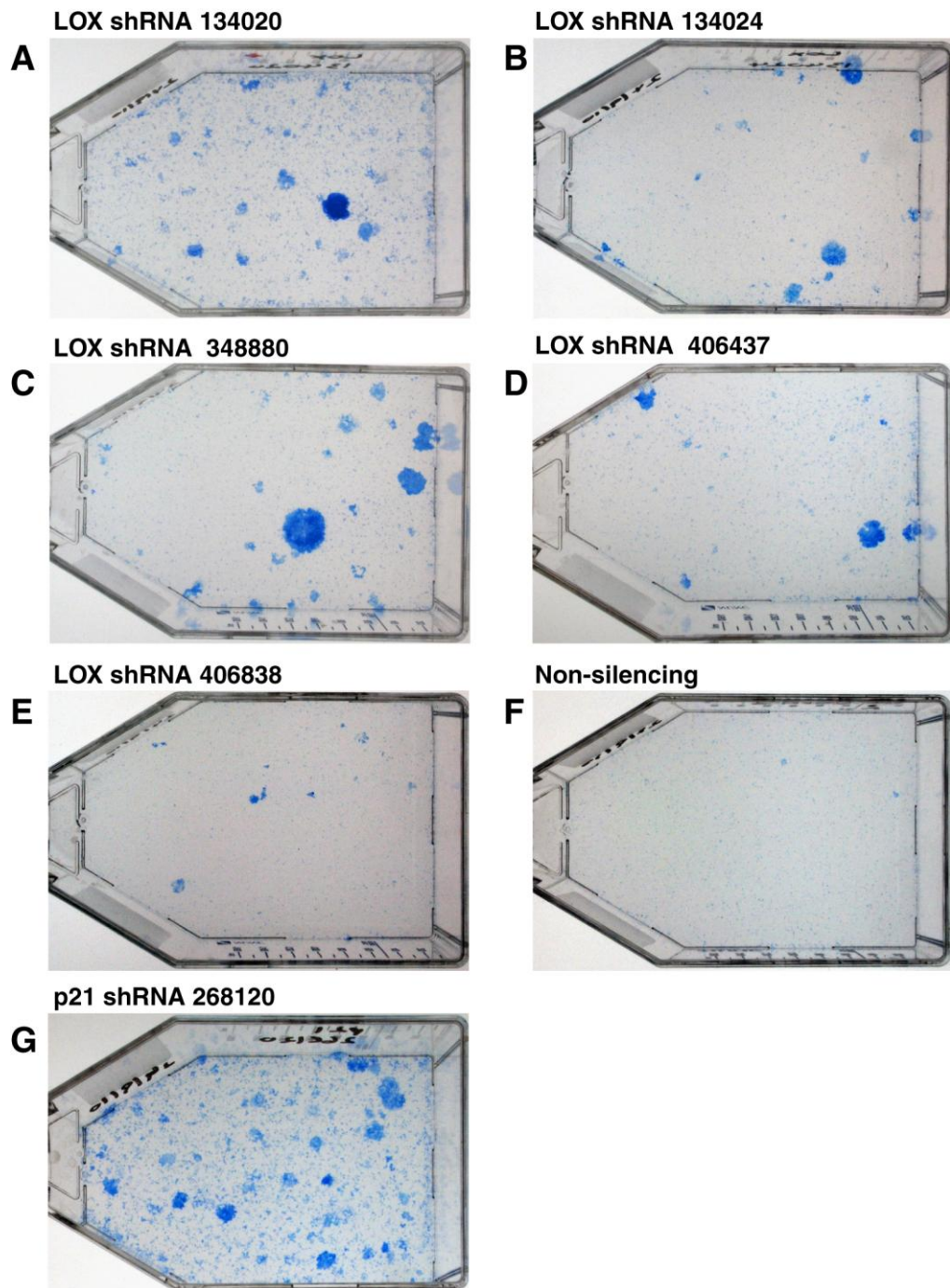
The IRF-1 shRNAs 133391, 133392, 133394, 386027, and 412360 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (**A**) 133391, (**B**) 133392 (**C**) 133394 (**D**) 386027 and (**E**) 412360. (**F**) Only very low background levels were detected in cells infected with the non-silencing control virus. (**G**) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

#### ***7.1.5.2 Lysyl Oxidase as a target of IRF-1 and its possible role in senescence***

Lysyl oxidase (LOX) was identified as an extracellular matrix enzyme which catalyses the cross-linking of collagens or elastin; it catalyses the oxidative deamination of peptidyl lysine to  $\alpha$ -aminoadipic- $\delta$ -semialdehyde (Kagan H.M., 1986; Kagan and Trackman, 1991; Pinnell and Martin, 1968). It has been demonstrated that there is an IRF response element in the LOX promoter, and that expression of LOX cDNA can suppress the transformed phenotype of ras-transformed IRF-1 null embryonic fibroblasts, inhibiting colony formation (Tan et al., 1996). Therefore, the effect of knock-down of LOX in the EcoR Clone 7 growth arrest was tested.

Five shRNAs targeting LOX were available from the Expression Arrest pGIPZ library and were tested in the EcoR Clone 7 complementation assay, namely V2LHS\_134020, V2LHS\_134024, V3LHS\_348880, V3LHS\_406437, and V3LHS\_406838. The experiment was set up in triplicate, and representative flasks are shown in Figure 7.10. Very few colonies were observed in the non-silencing control-infected cultures. In cultures infected with the hairpins 134020 and 348880 several healthy looking colonies of growing cells were detected, but many more colonies were detected in p21 shRNA infected cells. In cultures infected with the other three shRNAs targeting LOX only few colonies were detected. This suggests that LOX might act downstream of IRF-1 in senescence in EcoR Clone 7 cells, but this would need to be investigated further. Possibly, the LOX shRNA constructs were very inefficient, and therefore only weak rescue was achieved.





**Figure 7.10: Several LOX shRNAs abrogate the EcoR Clone 7 growth arrest**

The LOX shRNAs 134020, 134024, 348880, 406437, and 406838 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (**A**) 134020, (**B**) 134024 (**C**) 348880, and few colonies were detected in cells infected with the hairpins (**D**) 406437 and (**E**) 406838. (**F**) Only very low background levels were detected in cells infected with the non-silencing control virus. (**G**) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

## 7.2 Predicted cDNAs

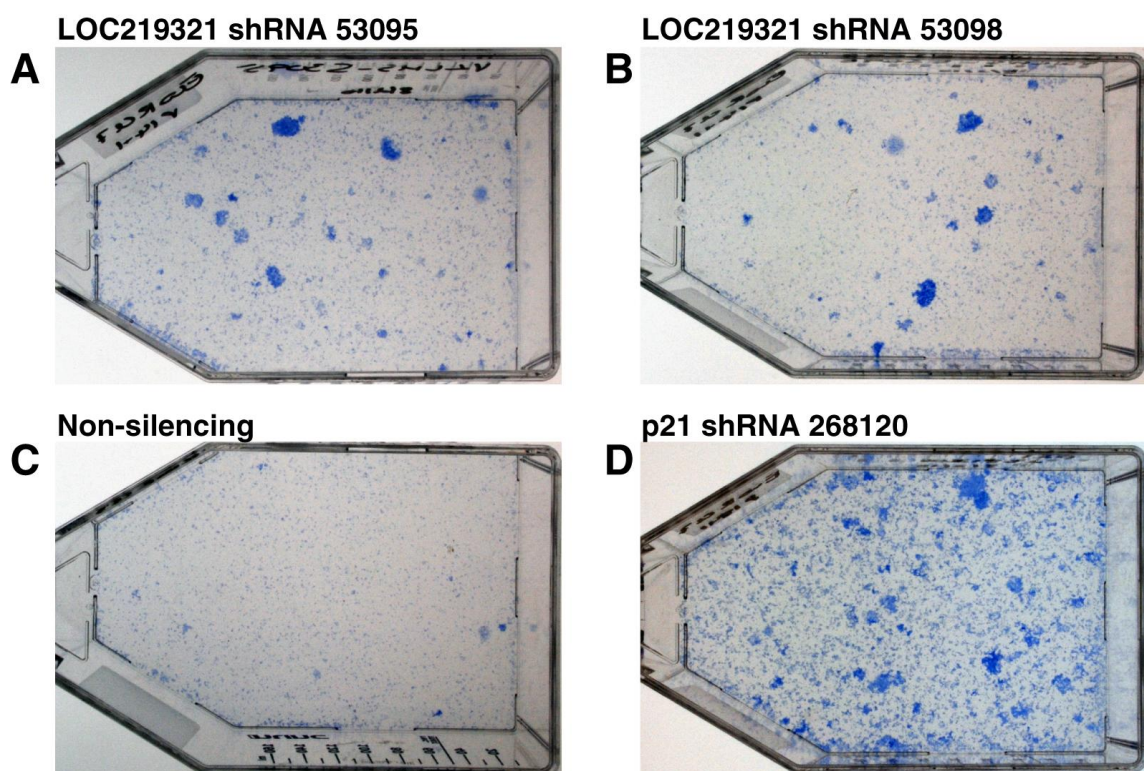
Not all of shRNAs detected in the screen and confirmed to work in a secondary screen were designed to target known genes. Some were designed to target expressed sequence tags (ESTs) within the database that had been used to design the shRNAs, and some were designed to target sequences which were predicted by computational analysis of genomic sequences. One of the shRNAs detected in the screen and confirmed to work in a secondary screen was designed to target a full length human cDNA described in ‘full-length long Japan’ (FLJ) collection of sequenced human cDNAs (Ota et al., 2004). For some of these predicted cDNAs only one hairpin was available, therefore in these cases the possibility of off-target effects could not be excluded. To confirm that these cDNAs were actually expressed in EcoR Clone 7 cells RT-PCR was performed. Furthermore, when cells were reseeded for the EcoR Clone 7 growth assay additional cultures were set up for RNA extraction, and RNA was harvested from EcoR Clone 7 cells infected with the hairpins and the non-silencing control, and RT-PCR was used to analyse knock-down of RNA levels by the hairpins.

### 7.2.1 V2LHS\_53095

The hairpin 53095, which was detected in pool 38 in the pSM2c screen, was designed to target LOC219321 which was predicted by computational analysis to be derived from the genomic sequence NT\_007933 (International Human Genome Sequencing Consortium).

#### 7.2.1.1 *Off-target effects*

One additional hairpin targeting LOC219321 was available from the Expression Arrest pGIPZ library, V2LHS\_53098, which was tested in the complementation assay. The experiment was set up in triplicate, and representative flasks are shown in Figure 7.11. Very low background levels were detected in non-silencing control-infected cultures. Multiple colonies of growing cells were detected in cultures infected with both LOC219321 shRNA constructs. This suggested that silencing LOC219321 was sufficient to overcome the conditional growth arrest of EcoR Clone 7 cells, and that it is therefore a mediator of cellular senescence in these cells.



**Figure 7.11: Two shRNAs targeting LOC219321 abrogate the EcoR Clone 7 growth arrest**

The LOC219321 shRNAs 53095 and 53098 were tested in the EcoR Clone 7 complementation assay. Colonies of growing cells were visible in cells infected with the hairpins (A) 53095 and (B) 53098. (C) Only very low background levels were detected in cells infected with the non-silencing control virus. (D) Abrogation of the growth arrest achieved by the p21 shRNA 268120.

### **7.2.1.2 Expression in EcoR Clone 7 cells at 34°C and 38°C**

To determine if the LOC219321 was expressed in EcoR Clone 7 cells primers were designed; Figure 7.12 shows LOC219321 and the primers. RNA was extracted from EcoR Clone 7 cells grown at 34°C and 38°C and was analysed for the expression of LOC219321 by RT-PCR. Unfortunately, the 331bp band was not detected at either temperature (data not shown). Bands were visible for the B2M ( $\beta$  2-Microglobulin) positive control, but this was only a control for the general PCR reaction, not for the reaction with the specific primers designed for the analysis. BLAST analysis indicated that the sequence tag did not have homology to genomic DNA in a continuous stretch of DNA but was interrupted by introns. Thus genomic DNA could not be used as a positive control as the sequence between these primers was too long due to these introns. As no positive control, i.e. a cell line which is known to express the EST, was available, new primers were designed which could be used to detect expression of the cDNA but which would also produce bands from genomic DNA, providing a positive control for the RT-PCR reaction (Figure 7.12). Using these primers RNA extracted from EcoR Clone 7 cells grown at the permissive and non-permissive temperature was analysed by RT-PCR and DNA extracted from EcoR Clone 7 cells grown at the permissive temperature by PCR (Figure 7.13 A). The expected 175bp was visible in the genomic DNA positive control. The band was also observed in the RT-PCR products from EcoR Clone7 cells grown at both temperatures. To confirm that these bands were not caused by genomic DNA contamination, the reaction was set up with and without the RT enzyme (RT- controls). No bands were observed in the RT- negative control PCRs. The LOC219321 band observed at 38°C appeared slightly weaker than that at 34°C, and no difference was detected between the B2M bands at the two different temperatures. These results showed that the mRNA LOC219321 was expressed in EcoR Clone 7 cells. Furthermore, the results suggested that it was very slightly down-regulated upon growth arrest.

### **7.2.1.3 Knock-down by shRNA**

Two shRNAs designed to target LOC219321, 53095 and 53098, were available from the Expression Arrest library. They were tested for their ability to knock-down

LOC219321 on the RNA level. The 175bp band was visible with the non-silencing control as well as with the 2 shRNA constructs, and no clear difference in intensity was observed between the non-silencing control and the shRNAs (Figure 7.13 B). RT-controls were set up to confirm that the bands were not produced by genomic DNA contamination. These results suggest that the shRNAs 53095 and 53098 did not silence LOC19321, but it is possible that RT-PCR was not sensitive enough to pick up knock-down if the level of reduction in mRNA was minimal. Furthermore, the shRNAs could inhibit translation of the mRNA rather than cause its degradation. The shRNAs appeared to abrogate EcoR Clone 7 growth arrest, and it is possible that this was due to knock-down of a related gene rather than LOC19321.

**LOC219321 (V2LHS\_53095)**

atggtcacatgccatggtgggattggagtaacgaactttgaaacagtgatagctcagg  
atgtccaggcttgaagctaagctatctggaaattctggagttgttaaagccacacgaca  
tggaccagaaggctctgaagaaactggtagaatccatcagtgaactgtcaagaactcc  
aaaaaaagtgagctagagtgtctcataagacttttccacagcttagtgggaagagctga  
tgaagattttgaaacactgggctagatctgttttttgcatttgacaaagaccatgata  
actacataaaagtaaaaagtggtggttaaaggattatcagtgtttcttcaaggaacgtat  
gaagaaaagctcaagtgtgaagatttctgtatgtgattactggcttagtaatatatag

F 5' cacatgccatggtgggattg

R 5' cactgataatcctttaaccactc

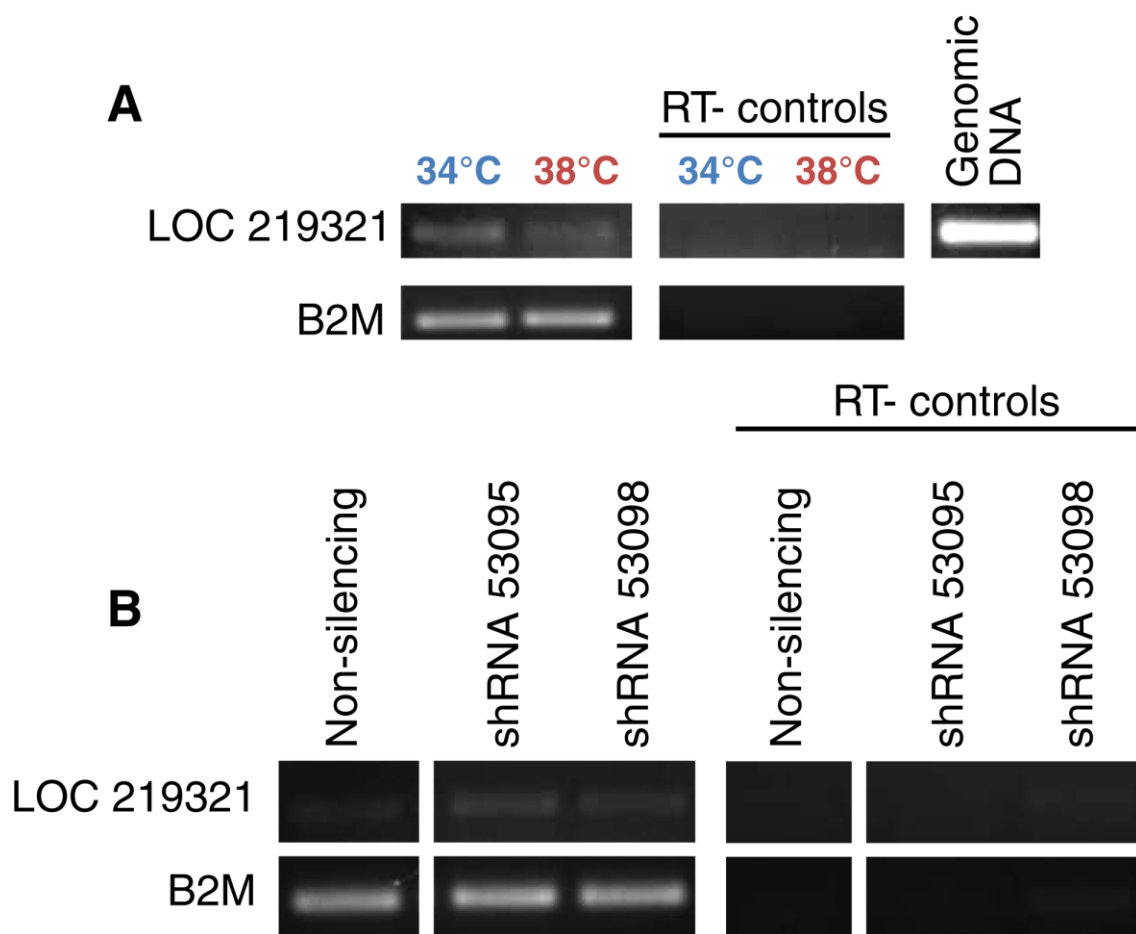
F 5' atggtcacatgccatggttg

R 5' agttcttgacagtttcactgatgg

**Figure 7.12: LOC219321 sequence and primers**

To determine if LOC219321 was expressed in EcoR Clone 7 cells, two sets of primers were designed.

The first set tested is depicted in red, and the second set (which would also produce bands from genomic DNA) highlighted in yellow



**Figure 7.13: Expression of LOC219321 in EcoR Clone 7 cells and knock-down by the shRNAs**

(A) LOC219321 was expressed in EcoR Clone 7 cells at 34°C and 38°C. (B) The hairpins 53095 and 53098 did not silence LOC219321

### **7.2.2 V2LHS\_25465**

The shRNA construct V2LHS\_25465 was detected in pool 2 in the Expression Arrest library pGIPZ screen. It was designed to target LOC343425 which was predicted by computational analysis to be derived from the genomic sequence NT\_004668 (International Human Genome Sequencing Consortium). Only one shRNA targeting LOC343425 was available, therefore off-target effects could not be ruled out.

#### ***7.2.2.1 Expression in EcoR Clone 7 cells at 34°C and 38°C***

To determine if LOC343425 was expressed in EcoR Clone 7 cells, RNA was extracted from EcoR Clone 7 cells grown at 34°C and 38°C and was analysed by RT-PCR. LOC343425 sequence and primers are depicted in Figure 7.14. Unfortunately, no band was detected at either temperature (data not shown). Bands were visible in the B2M positive control, but this was only a control for the general PCR reaction, not for the reaction with the specific primers designed for the analysis. Genomic DNA could not be used as a positive control as these primers were too far apart in genomic DNA as large introns were in between them. To enable the use of genomic DNA as a positive control for the primers and conditions of the PCR, new primers were designed which could be used to detect expression of the cDNA but which would also produce bands from genomic DNA (Figure 7.14). RNA extracted from EcoR Clone 7 cells grown at the permissive and non-permissive temperature was analysed by RT-PCR using these primers; genomic DNA extracted from EcoR Clone 7 cells grown at the permissive temperature was used as a positive control. A 203bp band was expected from this reaction; a band was detected which was approximately of this size, but unfortunately several bands were visible in the genomic DNA positive control (Figure 7.15). The same was observed for the RT-PCR products from EcoR Clone7 cells grown at both temperatures. No bands were detected in RT- controls. The band at 203bp might correspond to LOC343425, but more specific primers would have to be designed to determine its expression in EcoR Clone 7 cells.



**LOC343425 (V2LHS\_25465)**

atgcctattcaccacacactaccagcccctccaccaggaacagcatcagcaaccacg  
cagagaggagcccattgctgctgaaggaaaaccatttagagaccttaattccagtactg  
atgacatcaataccaccggaatcaatggaaaaataaaaacaaaaacagctctatctgta  
tctgactctgagcagagtgtggccttagccatggaagttgagcctctgtcctggaccct  
gggacccaccagggagcacgtcctgtcagataagccccagccagacagacgttgtcatt  
tgacttcagcacatcactctgatccagatcagttccaagctagacacccaatcatgagt  
ggaatttggtttgacacatacctggctgtgaaagccatgttctcgcccacacccttgcc  
cacttga

F 5' gcctattcaccacacactaccag

R 5' cgagaacatggctttcacagc

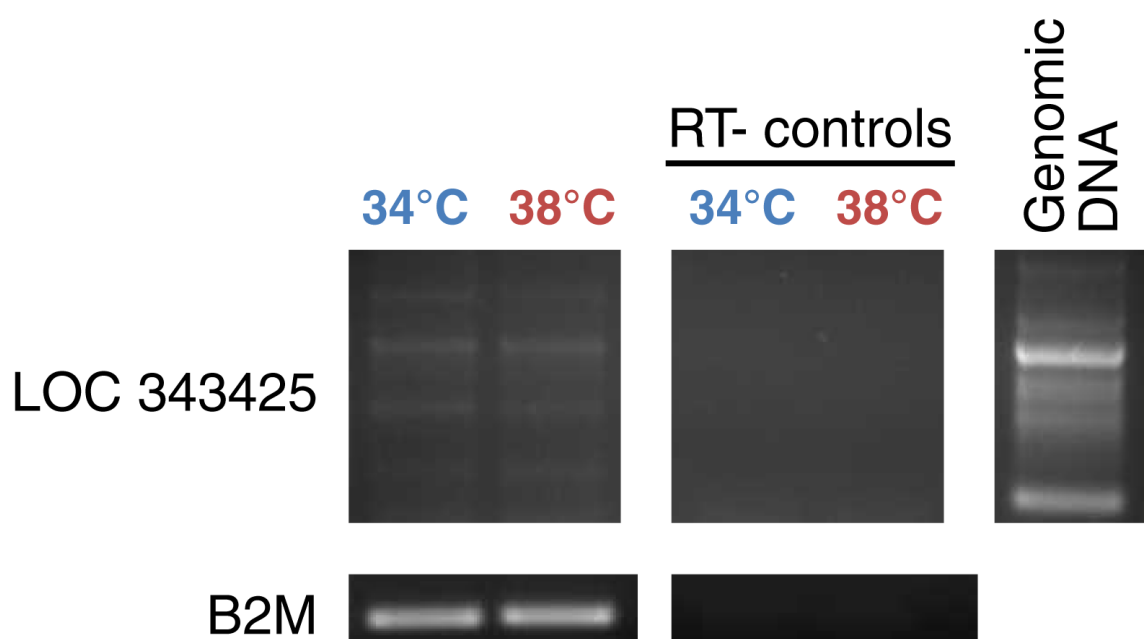
F 5' gcctctgtcctggaccctg

R 5' tcaagtgggcaagggtgg

**Figure 7.14: LOC343425 sequence and primers**

To determine if LOC343425 was expressed in EcoR Clone 7 cells, two sets of primers were designed.

The first set tested is depicted in red, and the second set (which would also produce bands from genomic DNA) highlighted in yellow



**Figure 7.15: Expression of LOC343425 in EcoR Clone 7 cells**

A 203bp was expected from this reaction; a band was detected which was approximately of this size, but unfortunately several bands were visible in the genomic DNA positive control. The same was observed for the RT-PCR products from EcoR Clone7 cells grown at both temperatures. No bands were detected in RT- controls. The band at 203bp might correspond to LOC343425, but more specific primers would have to be designed to determine its expression in EcoR Clone 7 cells.

### **7.2.3 V2LHS\_127153**

The shRNA 127153 was detected in pool 3 in the Expression Arrest pGIPZ screen. It was designed to target the cDNA LOC351347 which was predicted from the genomic sequence NT\_005403 (International Human Genome Sequencing Consortium). Off target effects could not be ruled out as only one hairpin targeting LOC351347 was available.

#### ***7.2.3.1 Expression in EcoR Clone 7 cells at 34°C and 38°C***

To determine if LOC351347 was expressed in EcoR Clone 7 cells, RNA was extracted from EcoR Clone 7 cells grown at the permissive and the non-permissive temperature and was analysed by RT-PCR; LOC351347 sequence and primers are depicted in Figure 7.16. Genomic DNA extracted from EcoR Clone 7 cells grown at the permissive temperature was used as a positive control for PCR conditions and primers. The expected 416bp band was detected with genomic DNA and with RNA extracted from cells grown at the permissive and non-permissive temperature, and the band at the non-permissive temperature appeared slightly weaker, whereas B2M control bands were of similar intensity. No bands were visible in the RT- controls (Figure 7.17 A). To confirm that the band corresponded to LOC351347 cDNA was gel-purified and sequenced using the same primers as used for the PCR reaction. The sequence detected was that of LOC351347 (Figure 7.18). These results confirm that LOC351347 was expressed by EcoR Clone 7 cells, and it appeared to be slightly down-regulated upon growth arrest at the non-permissive temperature.

#### ***7.2.3.2 Knock-down by shRNA***

Only the shRNA 127153 was available to target LOC35134, and it was tested for its ability to knock-down LOC351347 RNA levels. The 416bp band was detected with the non-silencing control, and it was also visible with the shRNA, and no clear difference in intensity was observed between the non-silencing control and the shRNA (Figure 7.17 B). No bands were detected in the RT- controls. These results suggest that the shRNAs 127153 did not silence LOC35134, but it is possible that RT-PCR was not sensitive

enough to pick up knock-down if the level of reduction in mRNA was small. Furthermore, the shRNA could inhibit translation of the mRNA rather than cause its degradation. The shRNA appeared to abrogate EcoR Clone 7 growth arrest, and it is possible that this was due to knock-down of a related gene rather than LOC35134.

**LOC351347 (V2LHS\_127153)**

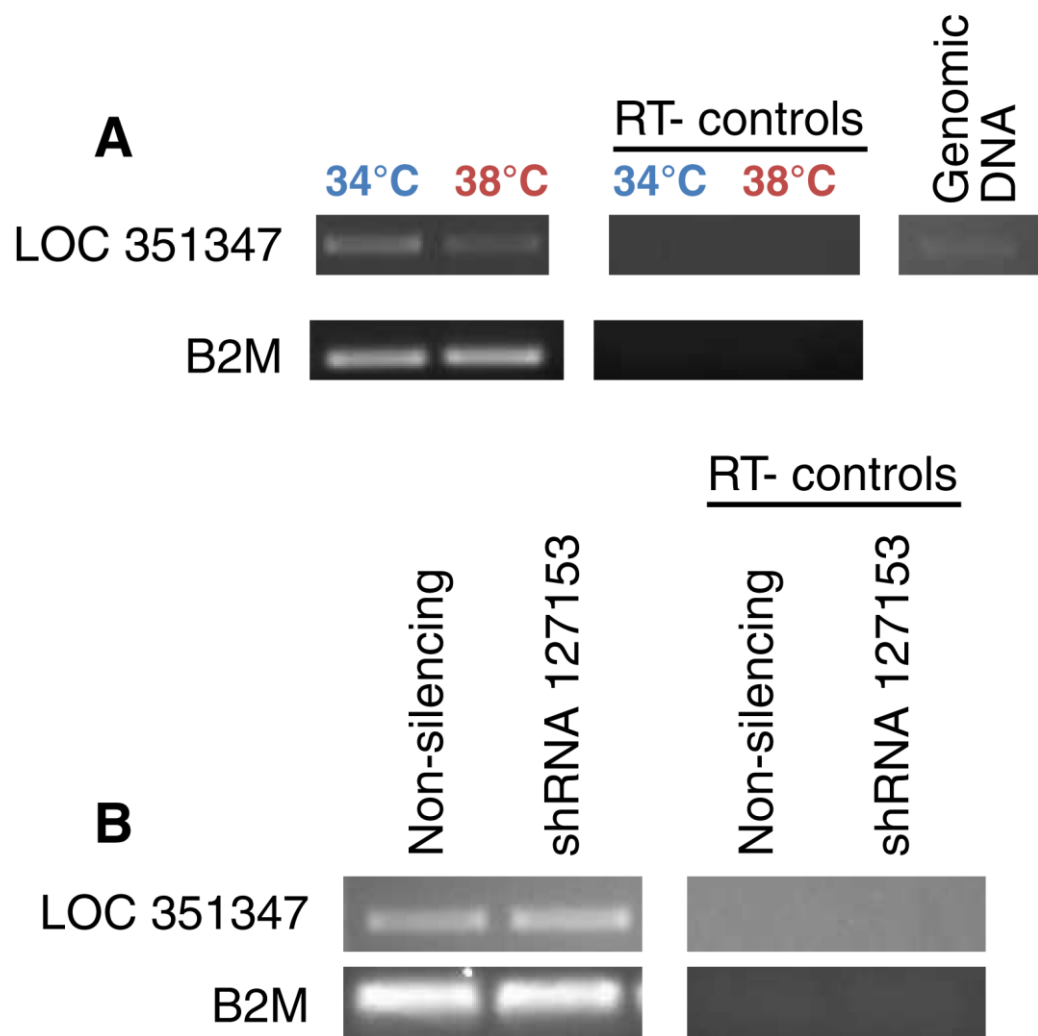
atggctcctcgcgtctctccggtgaatggccgagggctgggtggggctgcggtgggcag  
cggtggcctttctccttcacacacatacacatgccttccaaatgtgcatggcttgggat  
tcgcgtatattgtttaagagacgacggccacatcctttgttcggcggttcagcagactcc  
tccgagttccgggattttctcccgactgaggcgtctccgtctcagtactctcggaggaaa  
gcctcgagggggcagaactgccctctgttggtttcacggggctggaacctccctctccgca  
gccccaaagaatccggatggacccgcctttcccagaaccggagaccttggcctcctcagg  
ctcccctctgactcaaacaaaagccggaattggaaagaccacccctttgatgtgttcgc  
tgtaatcgacgtcctgctttcctcaaccgcccattcccttagccttcttagttga

F 5' ctcgcgtctctccggtgaatg

R 5' gacgtcgattacagcgaacacatc

**Figure 7.16: LOC351347 sequence and primers**

Primers were designed to determine if LOC351347 was expressed in EcoR Clone 7 cells



**Figure 7.17: Expression of LOC351347 in EcoR Clone 7 cells and knock-down by the shRNA**

(A) LOC351347 was expressed in EcoR Clone 7 cells at 34°C and 38°C. (B) The hairpin 127153 did not silence LOC351347

## A Sequence of the PCR reaction

nngngtntntncggtgaatggccgagggctgggtgggntgcggtgggcagcgggtggcc  
ttntccttnacacacacacacatgccttccaaatgtgcatggcttgggattcgcgatat  
ttgtttaanagacgacggccacatccttttggtcggcggttcagcagactcctccgagtt  
ccgggatttctcccgaactgagggcgtctccgtctcagtactctcggaggaaagcctcgag  
gggcagaactgccctctgttggtttcacggggctggaacctccctctccgcagcccaaag  
aatccggatggaccgcctttccagaaccggagaccttggcctcctcaggctccctc  
tgactcaaacaaaagccggaattggaaagaccaccctttgatgtgttcgctgtaatcn  
acgtca

## B Homo sapiens LOC351347 mRNA

atggctcctcgcgctctctccggtgaatggccgagggctgggtggggctgcggtgggcag  
cgggtggcctttctccttcacacacatacacatgccttccaaatgtgcatggcttgggat  
tcgcgatatttggttaagagacgacggccacatccttttggtcggcggttcagcagactcc  
tccgagttccgggatttctcccgaactgagggcgtctccgtctcagtactctcggaggaaa  
gcctcgaggggcagaactgccctctgttggtttcacggggctggaacctccctctccgca  
gcccagaatccggatggaccgcctttccagaaccggagaccttggcctcctcagg  
ctccctcttgactcaaacaaaagccggaattggaaagaccaccctttgatgtgttcgc  
tgtaatcgacgtcctgctttcctcaaccgcccattcccttagccttcttagttga

**Figure 7.18: LOC351347 PCR product sequence**

(A) Depicts the sequence of the PCR reaction. In red is the part of the PCR reaction sequence which matches the predicted sequence for LOC351347. (B) The sequence of Homo sapiens LOC351347 mRNA. Primers are revealed in blue. In red is shown the part of the LOC351347 predicted sequence which matches the sequence of PCR reaction.

#### **7.2.4 V2LHS\_68969**

The shRNA 68969 was designed to target the cDNA FLJ31301 (clone LIVER1000073), which is a full length human cDNA as described in the FLJ collection of sequenced human cDNAs (Ota et al., 2004). It was detected in pool 2 in the Expression Arrest pGIPZ screen and confirmed to achieve rescue in the secondary screen. Only one shRNA designed to target FLJ31301 was available, therefore off-target effects could not be ruled out.

##### ***7.2.4.1 Expression in EcoR Clone 7 cells at 34°C and 38°C***

To determine if FLJ31301 was expressed in EcoR Clone 7 cells, RNA was extracted from EcoR Clone 7 cells grown at 34°C and at 38°C and was analysed by RT-PCR using primers designed to produce a 199bp band from genomic DNA and cDNA; FLJ31301 sequence and primers are depicted in Figure 7.19. Genomic DNA extracted from EcoR Clone 7 cells grown at the permissive temperature was used as a positive control for PCR conditions and primers. The expected 199bp band was detected with genomic DNA; no band was detected with RNA extracted from cells grown at the permissive and non-permissive temperature, but B2M control bands were detected with these samples. No bands were visible in the RT- controls (Figure 7.20). This indicated that FLJ31301 was not expressed in EcoR Clone 7 cells incubated at 34°C or 38°C.



# FLJ31301 (V2LHS\_68969)

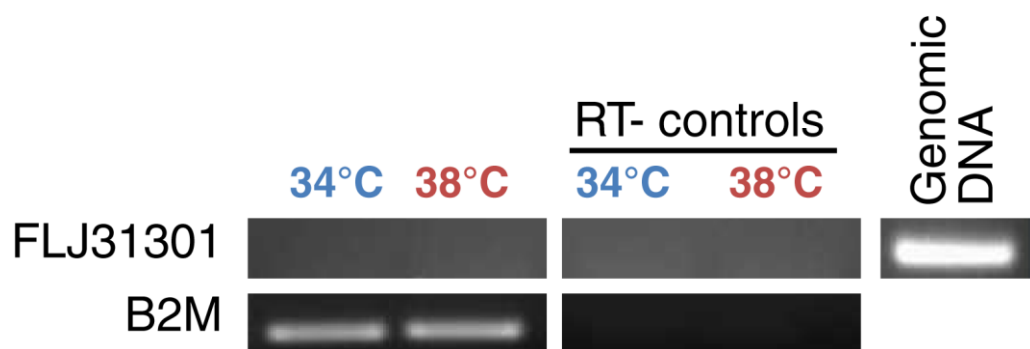
Caggcggtcacctggctcctagggcttttgtgctgtaggcctatatattccagctacttt  
tggaaaaaatctctctggaggctcaatgagcttgtcacatcagcctgcccagaggtaaa  
gcacatttctctgccctcacacgcctctcccttgctgtctttaacttaataatgcacg  
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ctctttactggactgtagaataatctctttgggtggtcttcctgtggtcttcccttccc  
tccactaaatctactctctattcagtatcaagaaagacttaatacagcagtgaatactct  
ccaccaagcccttgaccattgtcagctctctggcatcagatcctttccagtagggcaaa  
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ctgtaggcctatatattccagctacttttggaaaaaatctctctggaggctcaatgagct  
tgtcacatcagcctgcccagaggtaaagcacatttctctgccctcacacgcctctcca  
atgcccgtaacaccctctacaatgctgagcaagctcagatcctcctagtcttcatggca  
tccttctttttctttttccattgggtctaatactgattgaatttaaattttgcacatttg  
catgagtaattctcttgcataaatctctttctcttccctccttctgcatcactgggtc  
ttgtcctctctttactggactgtagaataatctctttgggtggtcttcctgtggtcttcc  
cttccctccactaaatctactctctattcagtatcaagaaagacttaatacagcagtga  
atactctccaccaagcccttgaccattgtcagctctctggcatcagatcctttccagta  
gggcaaagtgcactgcagccagctcatgccttc

F 5' cttgctgtctttaacttaataatgc

R 5' gagccagtgatggcagaagg

**Figure 7.19: FLJ31301 sequence and primers**

Primers were designed to determine if FLJ31301 was expressed in EcoR Clone 7 cells.



**Figure 7.20: Expression of FLJ31301 in EcoR Clone 7 cells**

FLJ31301 was not expressed in EcoR Clone 7 cells incubated at 34°C or 38°C.

### **7.2.5 V2LHS\_145373**

The shRNA 145373 was detected in pool 99 in the pSM2c screen, and it led to EcoR Clone 7 cells overcoming the conditional arrest in the secondary screen. The hairpin was designed to target LOC346321, which was predicted by automated computational analysis of the genomic sequence NT\_007758 (International Human Genome Sequencing Consortium). Only one shRNA designed to target LOC346321 was available, therefore off-target effects could not be ruled out.

#### ***7.2.5.1 Expression in EcoR Clone 7 cells at 34°C and 38°C***

To determine if LOC346321 was expressed in EcoR Clone 7 cells, RNA was extracted from EcoR Clone 7 cells grown at the permissive and the non-permissive temperature and was analysed by RT-PCR; LOC346321 sequence and primers are depicted in Figure 7.21. Genomic DNA extracted from EcoR Clone 7 cells grown at the permissive temperature was used as a positive control for PCR conditions and primers. A 901bp band was expected with genomic DNA and a 341bp band was expected from cDNA. The expected 901bp band was detected with genomic DNA. A faint band of approximately 901bp was also detected with RNA extracted from cells grown at the permissive and non-permissive temperature, where another two bands were also detected, one of approximately 650bp and one of approximately 550bp (Figure 7.22 A and B). But no 341bp band was observed with genomic DNA or cDNA. The band at 550bp was of the strongest intensity, the other two were a lot weaker, with the biggest one the least bright. No difference in intensity was detected between the permissive and the non-permissive temperature. No bands were visible in the RT- controls. To confirm that the bands corresponded to LOC346321, cDNA was gel-purified and sequenced using the same primers as used for the PCR reaction. Only the largest band was successfully sequenced, and it corresponded to LOC346321 but with additional exons (Figure 7.23). The other bands were meant to be sequenced again but as discussed because knock-down could not be demonstrated therefore this was not done. These results suggested that transcripts similar to LOC346321 were expressed in EcoR Clone 7 cells, which were different splice variants of LOC346321 that contained additional exons.

#### **7.2.5.2 Knock-down by shRNA**

The shRNA 145373 was tested for its ability to knock-down LOC346321 on the RNA level. All three bands were visible with the non-silencing control as well as with the shRNA construct 145373, and no clear difference in intensity was observed between the non-silencing control and the shRNA (Figure 7.22 C). No bands were detected in RT-controls. This suggested that the shRNA 145373 did not knock down the splice variants of LOC expressed in EcoR Clone 7 cells. But it is possible that RT-PCR was not sensitive enough to pick up knock-down if the level of reduction in mRNA was small. Furthermore, the shRNA could inhibit translation of the mRNA rather than cause its degradation. The shRNA appeared to abrogate EcoR Clone 7 growth arrest, and it is possible that this was due to knock-down of a related gene rather than LOC346321

**LOC346321 (V2LHS\_145373)**

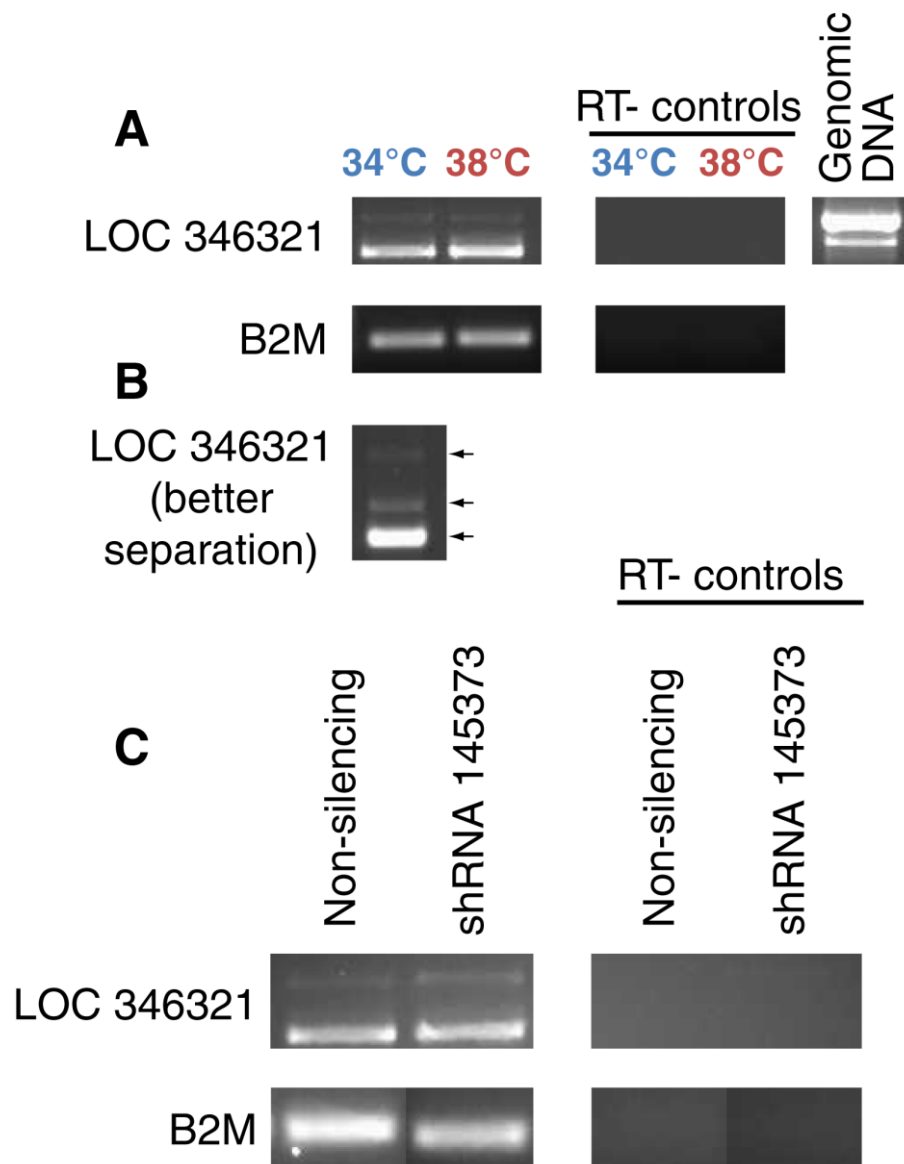
atgaccaagaagttgacgacagtcaggtcaggcttctcctccacggccatcggtccatt  
tcgtccggttacttgccgccaggtcacagctgctgcagctactccagtccaattcggca  
gacgctgggttaaagcctgccctaccactcataaaacagcagagggagctgctaagaga  
acatgccaaactgcagccttccaaagcccggaccgctcagcccagacagtcagccataa  
taaccccgagaagatcaggtactagtaaaagacctccaggcaagaggtctctccccccgt  
ggaaaggccctacatggtaattcttacaacacccgacggcagctaaacttacaggcctt  
ccctcctgggtccaacaatttattcaggttcttctcgaggaaagctgggtcattccctac  
ttttgctaacacctttcgctggcctgaaaatctgttgctctatatagacgaactggacc  
cccgctgggcctctgggtgtagatggaggactatatgagcacagaacttttatgtatcca  
gtagctaagatccgcattgccaggacccttaaaaccactgtcacagggttatccgactt  
agcctcctcaatccagtcagccgagaaagagcttgccagccagcttcaaccggcagctg  
accaggccaagtcctcccccttctcgtgggtttttaatttcagaagtacagtttctccgc  
cagcggatacaagaaatcaccagggtcactttcaaccagatgttacttcacccctacgt  
ccaactgccaacctctgatttaggcctctccccagtgacaccccttaa

F 5' ctcagcccagacagtcagcc

R 5' ggcaatgcggatcttagctactgg

**Figure 7.21: LOC346321 sequence and primers**

Primers were designed to determine if LOC346321 was expressed in EcoR Clone 7 cells.



**Figure 7.22: Expression of LOC346321 in EcoR Clone 7 cells and knock-down by the shRNA**

(A) LOC346321 was expressed in EcoR Clone 7 cells at 34°C and 38°C. (B) The hairpin 145373 did not silence LOC346321

## A Sequence of the PCR reaction

Nnnnnnnnnnnnnnnnnngnnnnnnnnnnnnnnntcnnnnnnnagaggtctctcccccggtggaagggcccctacntggtaattcttacnanaccgacggcagctaaacttacaggccttccctcctgggtaccatatttcccatcttaagagggcacctacacaacatcaggccacttgga  
ctgtcacttccctccccccaaccaaactgaaactctttaaatcaaataccgcatgaattctccatgtgacaggctccaacaattttatttcaggttcttctcgaggaaagctgggtcattccctacttttgctaacacctttcgctggcctgaaaatctgttgctctatatagacnaactgggtgtggcaaggctccctccagaactttcaccaacatgaagttcgctttgacaagccccctctcagactccctctcactgggttttttccctcncctgagaattggagttccacacaggcagntntcctgtagactactagccacggcagcatgcccgccagcagggtgccaggcncccatagctttcctaggtntaaaattntnttccctagggcccggtagaaaaaacctgcac  
tttgcttctctgtatgatcaaagtaactccaaatgcaataccagctgggtcaaagaaaatgtaggctgtccgtggcactgggtgcaatatccatgaggcattaattcgtactgaaaaaggatctgacccaatgttntangtcaatacctccactggaggatgggacggctttaacagatttaacctccaaatctcnacccttaggacccccgcnngggcctctggnnnagangaggactatanagcnnagannnnnnnn

## B Homo sapiens LOC346321 mRNA

atgaccaagaagttgacgacagtcaggtcaggtcttctcctccacggccatcggtccatttcgtccggttacttgccgccagggtcacagctgctgcagctactccaggtccaattcggcagacgtgggttaaagcctgccctaccactcataaaacagcagagggagctgctaagaga  
acatgccaaactgcagccttccaaagcccggaccgctcagcccagacagtcagccataataacccagaagatcaggtactagtaaaagacctccaggcaagaggtctctcccccggtggaaaggcccctacatggtaattcttacaacaccgacggcagctaaacttacaggccttcctcctgggtccaacaattttatttcaggttcttctcgaggaaagctgggtcattccctacttttgctaacacctttcgctggcctgaaaatctgttgctctatatagacgaactggacc  
cccgctgggcctctgggtgtagatggaggactatatgagcacagaacttttatgtatccagtagctaagatccgcattgccaggacccttaaaaccactgtcacagggttatccgacttagcctcctcaatccagtcagccgagaaagagcttgccagccagcttcaaccggcagctgaccaggccaagtcctcccccttctcgtgggtttttaatttcagaagtacagtttctccgc  
cagcggatacaagaaatcaccagggtcactttcaaccagatgttacttcacccctacgtccaactgcccaacc

**Figure 7.23: LOC346321 PCR product sequence**

(A) Depicts the sequence of the PCR reaction. In red is the part of the PCR reaction sequence which matches the predicted sequence for LOC346321. (B) The sequence of Homo sapiens LOC346321 mRNA. Primers are revealed in blue. In red is shown the part of the LOC346321 predicted sequence which matches the sequence of the PCR reaction.

### **7.2.6 V2LHS\_109096**

The shRNA 109096 was detected in pool 84 in the pSM2c screen, and it led to EcoR Clone 7 cells overcoming the conditional arrest in a secondary screen. This hairpin was designed to target LOC349975, predicted from the genomic sequence NT\_024524 by automated computational analysis (International Human Genome Sequencing Consortium). Unfortunately only this one hairpin was available against LOC349975, therefore off-target effects could not be ruled out by testing other hairpins.

#### ***7.2.6.1 Expression in EcoR Clone 7 cells at 34°C and 38°C***

To show expression of LOC349975 in EcoR Clone 7 cells RNA was extracted from cells grown at the permissive and at the non-permissive temperature and samples were analysed by RT-PCR; LOC349975 sequence and primers are depicted in Figure 7.24. Unfortunately, no band was detected at either temperature. Bands were visible in the B2M positive control, but this was only a control for the general PCR reaction, not for the reaction with the specific primers designed for the analysis. Genomic DNA could not be used as a positive control as these primers were too far apart in genomic DNA to run a PCR reaction. New primers were designed which could be used to detect expression of the cDNA but which were in closer proximity in genomic DNA to enable the use of genomic DNA as a positive control for the primers and conditions of the PCR (Figure 7.24). Samples were analysed by RT-PCR using these primers (Figure 7.25); genomic DNA extracted from EcoR Clone 7 cells grown at the permissive temperature was used as a positive control. The expected 146bp band was visible in the genomic DNA positive control. The band was not observed in the RT-PCR products from EcoR Clone7 cells grown at both temperatures, but B2M control bands were detected with these samples. No bands were observed in the RT- negative control PCRs. This indicated that LOC349975 was not expressed by EcoR Clone 7 cells at 34°C or 38°C. In the complementation assay, the shRNA 109096, designed to target LOC349975, appeared to abrogate EcoR Clone 7 growth arrest. It is possible that this was due to knock-down of a related gene rather than LOC349975.



**LOC349975 (V2LHS\_109096)**

Atggtagagacaagggcacatctaggaacacatagccaaggaacattagattcaagtcc  
agaactcggtttgagtgggcagagcctcaggaatacacttctggagaaagtccactgtc  
atagaaatgaaaaactggctcctccagatcacctttcagatccaattcgaggtttctcg  
catccgtcacagaaactgagccctaaatctgtgtctcctgggacagtcacactaattgg  
ccatcccagggttttgtttctcttaagggttcaggaagagatgttcccatatggaaatag  
gagccccaattactctaggtacttctgagtttctggccattgaagattctgttcaaata  
gaggttgagtggtcacgggagcaacctttccacggagcccggttcttcgtggatga

F 5' ggcacatctaggaacacatagccaag

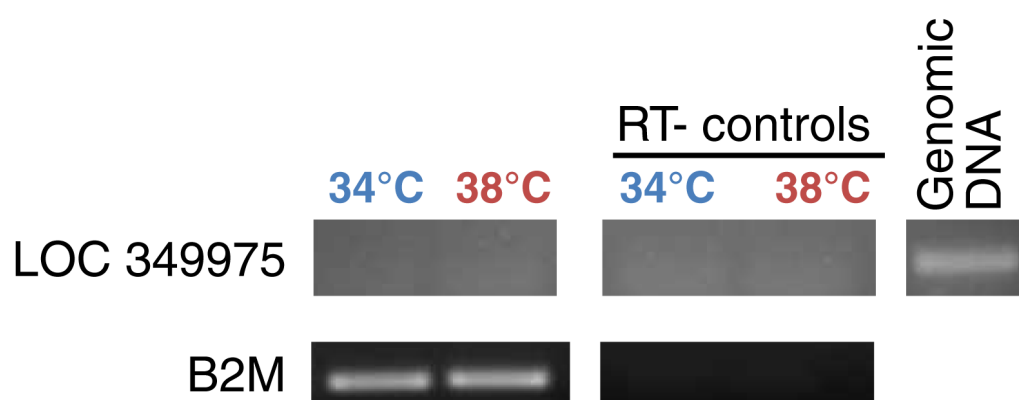
R 5' ggctccgtggaaagggttgctc

F 5' cagatccaattcgaggtttctcg

R 5' ctctatttccatatgggaacatc

**Figure 7.24: LOC349975 sequence and primers**

To determine if the LOC349975 was expressed in EcoR Clone 7 cells, two sets of primers were designed. The first set tested is depicted in red, and the second set (which would also produce bands from genomic DNA) highlighted in yellow



**Figure 7.25: Expression of LOC349975 in EcoR Clone 7 cells**

LOC349975 was not expressed in EcoR Clone 7 cells incubated at 34°C or 38°C.

### 7.3 Discussion

12 shRNAs identified in the pSM2c and pGIPZ screen had been validated in a secondary screen, and these shRNAs were designed to target the genes CCNL1, FOXA1, Rab23, SPOPL, IRF-1, BRCA1, LOC219321, LOC351347, LOC346321, LOC343425 FLJ31301 and LOC349975. To rule out off-target effects, several hairpins targeting the same gene were shown to abrogate growth arrest in the EcoR Clone 7 complementation assay for CCNL1, FOXA1, Rab23, SPOPL, IRF-1 and LOC219321. Additional hairpins targeting BRCA1 had previously been tested and shown not to abrogate the EcoR Clone 7 growth arrest (Figure 6.11). pGIPZ non-silencing control (scrambled) was used as a negative control, and the p21 shRNA V2LHS\_268120 as the positive control. The p21 construct 268120 was used instead of the construct 230370 as abrogation of growth arrest achieved with this construct is less efficient, and therefore a more comparable positive control when assaying targets whose effect is expected to be much weaker than that of 230370.

Furthermore, knock-down of target genes achieved by the shRNAs was demonstrated on the RNA level and on the protein level for CCNL1, FOXA1, and Rab23 and on the RNA level for SPOPL. Knock-down of IRF-1 by the shRNAs has not yet been analysed, but the fact that several hairpins targeting IRF-1 abrogated the growth arrest of EcoR Clone 7 cells proves that this was not caused by off-target effects but by knock-down of IRF-1. BRCA1 knock-down was not analysed. Because only one hairpin targeting BRCA1 was shown to abrogate the conditional arrest, knock-down of BRCA1 by this hairpin, and the failure to knock-down BRCA1 by the other hairpins which failed to abrogate the growth arrest, would need to be tested to demonstrate the abrogation of growth arrest was caused by knock-down of BRCA1.

For the predicted genes LOC219321, LOC351347 and LOC346321 expression in EcoR Clone 7 cells was demonstrated on the RNA level. Unfortunately, no knock-down was detected by the shRNAs targeting these genes when analysed by RT-PCR.

Expression of LOC343425 could not be demonstrated in EcoR Clone 7 cells, and it was shown that FLJ31301 and LOC349975 were not expressed in the cells. Therefore knock-down these genes by the shRNAs designed to target them was not investigated.

### 7.3.1 CCNL1

Three shRNAs targeting CCNL1 were shown to abrogate the EcoR Clone 7 growth arrest (Figure 7.1), indicating that the abrogation of growth arrest was caused by knock-down of CCNL1 and not by off-target effects. Furthermore, knock-down by the shRNAs was demonstrated (Figure 7.2). When analysed by western blotting, greater knock-down of CCNL1 was observed in cells grown at 38°C compared to cells grown at 34°C, suggesting that cells with better knock-down were selected for in the growth assay, i.e. this might be due to cells with greater knock-down growing better at the non-permissive temperature and therefore being selected for at 38°C. At 34°C, western blotting and quantitative RT-PCR demonstrated that even though CCNL1 levels were reduced by the shRNAs, a substantial amount of mRNA and protein still remained expressed. More efficient knock-down of CCNL1 might lead to better abrogation of the growth arrest; therefore more efficient constructs should be tested to determine if they improve results.

#### ***7.3.1.1 CCNL1, cell cycle control and cancer***

Cyclin L1 was first identified as ania-6 in a screen for genes whose expression rapidly increases in response to stimulation of striatal dopamine D1 receptors (Berke et al., 1998). Furthermore, it was identified as a regulatory subunit of the cyclin-dependent kinase CDK11-p110 (Berke et al., 2001).

The results presented in this thesis suggest that CCNL1 is involved in the induction of senescence in human breast epithelial cells. In the Hanahan and Weinberg paradigm of tumourigenesis, overcoming finite proliferative potential is one of the six hallmarks of cancer (Hanahan and Weinberg, 2011). If CCNL1 is required for the induction of senescence, then lack of CCNL1 could increase the risk of cancer. Down-regulation of CCNL1 has been connected to breast cancer by several studies. Ductal carcinoma in situ (DCIS) is the most common type of pre-invasive breast cancer, and CCNL1 has been shown to be down-regulated in DCIS compared to normal breast epithelium (Abba et al., 2004). CCNL1 has also been shown to be down-regulated in epithelium from oestrogen receptor-positive (ER positive) breast cancers compared to reduction mammoplasty controls (Tripathi et al., 2008). Both these findings indicate that loss of

CCNL1 might be involved in development of breast cancer. Park and colleagues identified genes whose expression changes upon primordial to primary follicle transition in the mouse ovary (Park et al., 2005). After ovarian follicles are formed they enter a resting stage which persists for some time (Fortune et al., 2000). They then stay in this resting stage until they either degenerate or are induced to enter growth. This induction of a phase of proliferation and differentiation is known as primordial to primary follicle transition. Park et al found that CCNL1 was down-regulated in dividing primary follicles compared to non-dividing primordial follicles, together with other genes involved in cell cycle control such as Wee1 and Lats2. This finding indicates that CCNL1 is an inhibitor of cell growth, as it is down-regulated upon the induction of growth, which correlates with the role identified in this thesis of CCNL1 as a mediator of cellular senescence.

### **7.3.2 FOXA1**

Three shRNAs targeting FOXA1 were shown to abrogate the EcoR Clone 7 growth arrest (Figure 7.3), indicating that the abrogation of growth arrest was caused by knock-down of FOXA1 and not by off-target effects. Furthermore, knock-down by the shRNAs was demonstrated (Figure 7.4). Knock-down at mRNA level was only to about 50 percent, whereas it appeared much more efficient when analysed by western blotting. This could be due to the shRNA constructs inhibiting translation in addition to causing degradation of FOXA1 mRNA as shRNAs can cause knock-down by inhibition of translation [reviewed by (Pekarik, 2005)]. It is also possible that the FOXA1 antibody was not sensitive enough to detect lower levels of FOXA1.

#### ***7.3.2.1 FOXA1, cell cycle control and cancer***

Forkhead box protein A1 is a member of the forkhead class of DNA-binding proteins and was first discovered by Costa and colleagues as a transcription factor in hepatocytes, named HNF-3 $\alpha$  (Costa et al., 1989).

Findings presented in this thesis suggest a role of FOXA1 in the induction of cellular senescence in human breast epithelial cells. One way it could exert this function is by

activating p27, a member of the Cip/Kip family of CDKIs which inhibit cell cycle progression (Russo et al., 1996). A FOXA1 binding site has been identified in the p27 promoter within the BRCA1-responsive element, and FOXA1 can activate the promoter in conjunction with BRCA1 or alone but more strongly in conjunction with BRCA1 (Williamson et al., 2006). Furthermore, Williamson and colleagues found that the half-life of FOXA1 was increased by co-expression with BRCA1. Several p27 shRNAs were sufficient to overcome the conditional growth arrest of EcoR Clone 7 cells (Figure 7.5), which is consistent with the hypothesis that FOXA1 induces senescence arrest by activating p27 expression in the EcoR Clone 7 cells.

Exogenous expression of FOXA1 has been shown to inhibit growth of some breast cancer cell lines, namely MCF-7, MDA-MB-231, and SKBR3, and increased FOXA1 levels led to a reduction in growth stimuli (Wolf et al., 2007). Wolf and colleagues also showed that in ER-positive MCF-7 cells FOXA1 could increase activity of the p27 promoter, in accordance with previous findings, and inhibit the ER pathway. These findings are consistent with the finding concerning FOXA1 presented in this thesis, and strongly suggest a role of FOXA1 as a mediator of cellular senescence.

In contrast, others have found that FOXA1 is highly expressed in ErbB2-positive cell lines and some ErbB2-negative cell lines, and that knock-down of FOXA1 by RNAi could suppress proliferation in ErbB2-negative FOXA1-positive cell lines (Yamaguchi et al., 2008). These opposing findings might be due to cell-type specific functions of FOXA1.

It has been shown that FOXA1 expression correlates with a good outcome in breast cancer, and FOXA1 expression was inversely related with tumour size, histological grade, Nottingham Prognostic Index, Lymph vascular invasion, lymph node stage, expression of basal cytokeratins (Cytokeratin 14 and cytokeratin 5/6), P-cadherin expression and human epidermal growth factor receptor-2 (HER-2) over-expression (Albergaria et al., 2009; Habashy et al., 2008). The inverse relation of FOXA1 expression and tumour size is consistent with a role of FOXA1 in suppressing cell growth.

FOXA1 is expressed mostly in breast cancer cell lines that express ER $\alpha$  and FOXA1 expression has been shown to correlate with ER $\alpha$  positivity and luminal type A phenotype breast cancers (Badve et al., 2006). In contrast to its growth-inhibitory effects, FOXA1 is a pioneer factor required for the function of the ligand-activated transcription factor ER $\alpha$ ; it binds to chromatinized DNA and opens the chromatin, increasing binding of ER $\alpha$  to target genes, thereby promoting oestrogen dependent proliferation; It is required for expression of approximately 50% of ER $\alpha$ :oestrogen-regulated genes (Carroll et al., 2005; Laganier et al., 2005). The association of FOXA1 with a favourable outcome, its ability to activate p27 and inhibit cell proliferation, and its ability to promote oestrogen dependent proliferation suggest multiple roles for this protein in breast cancer.

### **7.3.2.2 FOXA1, BRCA1 and p27**

It was shown that several p27 shRNAs were sufficient to overcome the EcoR Clone 7 growth arrest (Figure 7.5). This result is consistent with the hypothesis that FOXA1 induces senescence arrest by activating p27 expression in the EcoR Clone 7 cells, but it does not prove that FOXA1 inhibition leads to abrogation of the growth arrest due to a lack of activation of p27.

If FOXA1 acts via p27, it would be reasonable to expect p27 knock-down to bypass the growth arrest more efficiently than FOXA1 knock-down. But in the complementation assay, FOXA1 knock-down appeared to abrogate the growth arrest more efficiently. Knock-down by the p27 constructs was not determined, therefore the weaker abrogation of the growth arrest could have been caused by inefficient knock-down by the shRNAs. It is also possible that FOXA1 does not only act via p27 but also has additional effects which contribute to the abrogation of the growth arrest, or that it acts on a different pathway altogether. Further experiments are required to test this hypothesis, such as assays to determine p27 promoter activation by FOXA1.

It could be tested if knock-down of FOXA1 in EcoR Clone 7 cells which express a constitutively active p27 could abrogate growth arrest in the complementation assay. Also, it could be tested if silencing of FOXA1 further improves abrogation of the growth arrest in EcoR Clone 7 cells in which p27 has been completely knocked-down.

If so, this would indicate that FOXA1 does not act solely or not at all via p27 to mediate induction of senescence in EcoR Clone 7 cells.

As discussed above, FOXA1 can activate the p27 promoter in conjunction with BRCA1 or alone but more strongly in conjunction with BRCA1 (Williamson et al., 2006). A BRCA1 shRNA which can overcome the EcoR Clone 7 growth arrest had been detected in the screen, suggesting that knock-down of BRCA1 can abrogate the growth arrest. Therefore, FOXA1 and BRCA1 shRNAs should be tested in combination in the complementation assay, to analyse if this would lead to a greater abrogation of the growth arrest than knock-down of either gene alone.

### **7.3.3 Rab23**

Five shRNAs targeting Rab23 were shown to abrogate the EcoR Clone 7 growth arrest (Figure 7.6), indicating that the abrogation of growth arrest is caused by knock-down of Rab23 and not by off-target effects. Furthermore, knock-down by the shRNAs was demonstrated (Figure 7.7).

#### ***7.3.3.1 Rab23, cell cycle control and cancer***

Rab23 is a member of the Ras-related Rab family of small GTPases and was identified by Oikkonen and colleagues (Oikkonen et al., 1994).

The work presented in this thesis indicates that Rab23 acts as a mediator of cellular senescence in human breast epithelial cells. This is in accordance with the finding by Zeng and colleagues that Rab23 over-expression inhibited the proliferation of the breast cancer cell lines MDA-MB-231, Bcap-37 and MCF-7 when measured by colony formation assay, BrdU labelling and MTT assay; Rab23 silencing led to increased proliferation (Chao et al., 2009).

Rab23 can act as a negative regulator of sonic hedgehog signalling (Eggenschwiler et al., 2001; Eggenschwiler et al., 2006; Wang et al., 2006), and hedgehog signalling has been shown to promote proliferation in certain cancers (Yuan et al., 2007; Zhang et al., 2007). The Hedgehog signalling pathway has been found to be constitutively activated in many cancers particularly basal cell carcinoma; mutations in either patched (the



transmembrane spanning shh receptor) or smoothened (transmembrane spanning protein that is the central transducer of the Hedgehog signalling) are present in >70% of all basal cell carcinomas. Rab23 has been shown to act as a negative regulator of sonic hedgehog signalling and to regulate the Gli family of transcription factors, which are activated by the sonic hedgehog signal transduction cascade, and smoothened levels (Eggenchwiler et al., 2001; Eggenchwiler et al., 2006; Evans et al., 2003; Evans et al., 2005; Wang et al., 2006). By this action Rab23 could exert an anti-proliferative effect in EcoR Clone 7 cells and other cell types. To investigate this, the effect of Rab23 on shh signalling in EcoR Clone 7 cells should be analysed. Furthermore, it could be tested if over-expression of shh signalling members could abrogate the EcoR Clone 7 growth arrest in the complementation assay. Shh shRNAs were introduced into EcoR Clone 7 cells to analyse if growth of the cells at 34°C would be affected by knock-down of shh, but no difference was detected (data not shown). But it is important to mention that these are only very preliminary observations; growth was only analysed by methylene blue staining which would not visualize minor differences, and knock-down was not analysed.

On the other hand, over-expression of Rab23 has been observed in some cancers, and it has been shown that knock-down of Rab23 can inhibit growth in hepatocarcinoma cells (Huang et al., 2010; Liu et al., 2007), which contrasts the findings presented in this thesis. Possibly, the effects of Rab23 on cell proliferation might be cell-type dependent.

#### **7.3.4 SPOPL**

Three shRNAs targeting SPOPL were shown to abrogate the EcoR Clone 7 growth arrest (Figure 7.8), indicating that the abrogation of growth arrest is caused by knock-down of SPOPL and not by off-target effects. Furthermore, knock-down by the shRNAs was demonstrated by quantitative RT PCR (Figure 7.8 F). Surprisingly, knock-down by the shRNA 14455 which had been detected in the screen was the least efficient, only knocking the mRNA levels down to approximately 70%, whereas the other two constructs knocked mRNA down to about 30%. Knock-down should be determined at the protein level, as it is possible that the hairpins inhibit translation of the mRNA rather

than just causing degradation, and therefore knock-down at the protein level might be better than that observed at the mRNA level.

Substantial amounts of mRNA and protein still remained expressed in cells infected with the shRNAs. More efficient knock-down of SPOPL might lead to better abrogation of the growth arrest; therefore more efficient constructs should be tested to determine if they would improve results.

#### ***7.3.4.1 SPOPL, cell cycle control and cancer***

Data presented in this thesis indicate that SPOPL acts as a mediator of cellular senescence in human breast epithelial cells. Not much is known about the function of SPOPL, also known as Roadkill homolog 2.

Perhaps, SPOPL, a SPOP (Roadkill homolog 1) paralog, could negatively regulate cellular proliferation by inhibiting hedgehog signalling, which has been shown to promote proliferation in certain cancers (Yuan et al., 2007; Zhang et al., 2007). SPOP has been demonstrated to act as a negative regulator of hedgehog signalling by degradation of *Cubitus interruptus* (Ci), and inactivation of SPOP leads to Ci accumulation and enhanced hedgehog signalling, whereas over-expression of SPOPL causes Ci down-regulation and inhibition of hedgehog signalling (Kent et al., 2006; Zhang et al., 2006). It is conceivable that SPOPL could also negatively regulate hedgehog signalling, and thereby cell proliferation.

SPOP has also been demonstrated to have an effect on p53 dependent transcription. It inhibits Daxx (death-domain associated protein) activity and thereby destabilizes Mdm2, which in turn leads to increased p53 levels (Kwon et al., 2006). Daxx has been shown to act as a repressor of p53, as it stabilizes the E3 ubiquitin ligase Mdm2 (Tang et al., 2006; Zhao et al., 2004), which promotes p53 ubiquitination and degradation (Michael and Oren, 2003). If, like SPOP, SPOPL also negatively regulates Daxx activity, it could exert its growth inhibitory function by increasing levels of p53.

Therefore, the effect of SPOPL knock-down on shh signalling and p53 levels should be investigated to determine if SPOPL mediates growth arrest via one of these pathways in EcoR Clone 7 cells.

### **7.3.5 IRF-1**

Five shRNAs targeting IRF-1 were shown to abrogate the EcoR Clone 7 growth arrest (Figure 7.9), indicating that the abrogation of growth arrest was caused by knock-down of IRF-1 and not by off-target effects. Knock-down of IRF-1 by the shRNAs has not been analysed, and this should be done by quantitative RT-PCR and by western blotting ideally at 34°C and 38°C. But the fact that 5 individual IRF-1 shRNAs overcame the conditional arrest demonstrated that this was due to IRF-1 knock-down, not off-target effects.

#### ***7.3.5.1 IRF-1, cell cycle control and cancer***

The work presented in this thesis indicates that IRF-1 acts as a mediator of cellular senescence in human breast epithelial cells. IRF-1 is a member of the IRF family of transcription factors, whose DNA binding domain recognizes a similar DNA sequence, IRF-E, in the promoters of many IFN inducible genes (Mamane et al., 1999; Tanaka et al., 1993). IRF-1 was the first member identified (Miyamoto et al., 1988). IFNs have antiviral and immunomodulatory functions (Balkwill, 1979), and can inhibit cell proliferation *in vivo* and *in vitro*, and are therefore known as negative growth factors (Gresser et al., 1973; Gresser et al., 1979; Gresser et al., 1983). It has been shown that IRF-1 can induce p21 in cooperation with p53 (Tanaka et al., 1996); IRF-1 binds to IRF-E elements in the human p21 promoter (Coccia et al., 1999). Others have shown that IRF-1 up-regulates p21 in p53 null cells but not in p53 wild type cells, and that its growth inhibitory effects are stronger in p53 null cells (Armstrong et al., 2003). Furthermore, IRF-1 up-regulates p21 in the breast cancer cell lines MDA-MB-468 and SK-BR-3 in which p53 is mutated (Pizzoferrato et al., 2004). IRF-1<sup>-/-</sup> embryonic fibroblasts (EFs) from mice with a null mutation in the IRF-1 gene can be transformed by expression of an activated Ras (Tanaka et al., 1994). In Ras-induced growth suppressed myeloid cells IRF-1 is up-regulated, and the Ras-induced growth suppression is dependent on IRF-1 (Passioura et al., 2005). IRF-expression has also been shown to be up-regulated by drug-induced cellular senescence, such as BrdU, in

normal and in cancer cells (Novakova et al., 2010). It has been demonstrated that the HPV16 oncoprotein E7 can inhibit the tumour suppressor function of IRF-1 through an interaction between the pRb binding portion of E7 and IRF-1 (Park et al., 2000). The inactivation or loss of IRF-1 in several tumours further supports a role in tumour suppression; for example IRF-1 expression was shown to be lost in human breast-carcinoma (Doherty et al., 2001). IRF-1 has also been shown to regulate DNA damage-induced cell cycle arrest in cooperation with the tumour suppressor p53 through transcriptional activation of p21 (Tanaka et al., 1996). The data presented in this thesis further support the role of IRF-1 in cellular senescence and tumour suppression, specifically in breast epithelial cells.

#### ***7.3.5.2 Lysyl Oxidase as a target of IRF-1 and its possible role in senescence***

It has been demonstrated that there is an IRF response element in the LOX promoter, and that expression of LOX can suppress the transformed phenotype of Ras-transformed IRF-1 null embryonic fibroblasts, as shown by inhibition of colony formation (Tan et al., 1996). Therefore, the effect of knock-down of LOX in the EcoR Clone 7 growth arrest was tested. In cultures infected with two LOX hairpins, namely 134020 and 348880, several healthy looking colonies of growing cells were detected, suggesting that LOX knock-down might be sufficient to overcome the EcoR Clone 7 growth arrest. This suggests that LOX might act downstream of IRF-1 in senescence in EcoR Clone 7 cells, but this would need to be investigated further. Knock-down by the LOX shRNAs needs to be analysed; possibly, some of the LOX shRNA constructs were very inefficient, and therefore only weak rescue was achieved.

#### **7.3.6 Predicted cDNAs**

For the predicted genes LOC219321, LOC351347 and LOC346321 expression in EcoR Clone 7 cells was demonstrated on the RNA level (Figures 7.13, 7.17 and 7.22 respectively). Unfortunately, no knock-down was detected by the shRNAs targeting these genes when analysed by RT-PCR (Figures 7.13, 7.17 and 7.22 respectively). This is in contrast to these shRNAs overcoming the EcoR Clone 7 conditional arrest. It is

possible that these shRNAs inhibit translation of the mRNA rather than cause degradation; therefore they might achieve knock-down at the protein level but not at the mRNA level. It is also possible that RT-PCR was not sensitive enough to pick up knock-down if the level of reduction in mRNA was minimal. Another possibility is that abrogation of the growth arrest was caused by off-target effects, especially in the case of LOC351347 and LOC346321 as only one construct designed to target each of these predicted genes was available and therefore off-target effects could not be ruled out. In the case of LOC219321 off-target effects were less likely, since two LOC219321 shRNAs were shown to abrogate the EcoR Clone 7 growth arrest, namely 53095 and 53098 (Figure 7.11).

It was shown that FLJ31301 (Figure 7.20) and LOC349975 (Figure 7.25) were not expressed in EcoR Clone 7 cells. B2M control bands were detected with the samples, demonstrating that the RNA extracts were fine, and genomic DNA was used as a control to confirm that the PCR and the primers worked. As it was shown that these genes were not expressed, knock-down by the shRNAs designed to target them was not investigated. Since these genes were not expressed in EcoR Clone 7 cells, abrogation of the growth arrest was most likely caused by off-target effects. It could be analysed what gene sequences these shRNAs are similar to and shRNAs targeting such genes should be tested in the complementation assay.

Expression of LOC343425 could not be demonstrated in EcoR Clone 7 cells as the primers designed produced multiple bands (Figure 7.15). More specific primers need to be designed to analyse expression of this predicted gene in EcoR Clone 7 cells.

Surprisingly, both LOC219321 and LOC351347 appeared to be slightly down-regulated upon growth arrest (Figures 7.13 and 7.17 respectively), which is in contrast to knock-down of these predicted genes causing abrogation of the growth arrest. This further supports that abrogation of the growth arrest by the hairpins designed to target these mRNAs (53095 and 53098, and 127153 respectively) might have been caused by off-target effects.

## 8 Final discussion

### 8.1 Summary and discussion of results

This thesis presents the characterisation of a conditionally immortalised human breast epithelial cell line followed by two shRNA screens in this cell line which identified 6 genes which mediate growth arrest in these cells and a further 6 shRNAs which abrogate the conditional growth arrest.

226L 8/13 cells were derived from human breast luminal epithelial cells. First, it was demonstrated that 226L 8/13 cells express the epithelial markers  $\beta$ -catenin and E-cadherin. Most cancers are derived from epithelial cells, and overcoming the finite proliferative potential, i.e. senescence, is required for the development of cancer (Hanahan and Weinberg, 2011). Many studies are done in fibroblasts, but cancer is cell type specific, and the fact that 226L 8/13 cells are epithelial makes them a highly relevant model to study senescence.

Following the finding that 226L 8/13 cells expressed epithelial markers they were analysed for expression of the luminal epithelial markers cytokeratin 18 and cytokeratin 19, and the myoepithelial markers cytokeratin 14 and  $\beta$ -4 integrin. 226L 8/13 cells were positive for the luminal marker cytokeratin 18 and the basal markers cytokeratin 14 and  $\beta$ -4 integrin, but were negative for the luminal marker cytokeratin 19. Originally, luminal cells were isolated to generate the 226L 8/13 cells, but culturing the cells *in vitro* and immortalisation of the cells could have caused expression of such markers to change (Bartek et al., 1991; Moll et al., 1982; Spancake et al., 1999; Taylor-Papadimitriou et al., 1989). However, it has been shown that some luminal epithelial cells in the breast are cytokeratin 19 negative (Bartek et al., 1985), and that some keratin 14 expression is seen in luminal cells in the very large ducts (Taylor-Papadimitriou et al., 1989). 226L cells could have been derived from cells with such an expression profile.

The next step was to determine the growth characteristics of 226L 8/13 cells. To immortalize breast luminal epithelial cells, Mike O'Hare and colleagues found that hTERT was not sufficient but additional activities were required, which could be provided by SV40 LT antigen. 226L 8/13 cells were conditionally immortalized by

introduction of hTERT and a temperature sensitive LT antigen, U19tsA58 LT, which is active at 34°C but becomes inactive at 38°C. It was demonstrated that 226L 8/13 cells grow at 34°C, at which temperature the temperature-sensitive LT is active, and that they cease to grow at 38°C, where the ts LT is inactive. Furthermore, 226L 8/13 cells incubated at the non-permissive temperature for two weeks were positive for senescence associated  $\beta$ -galactosidase, a marker widely recognised of cellular senescence (Dimri et al., 1995), demonstrating that the arrest at 38°C was senescence.

It was then demonstrated that reconstitution of wt LT activity was sufficient to overcome the conditional growth arrest of 226L 8/13 cells at 38°C. This indicated that the 226L 8/13 system could be used to dissect the pathways underlying the induction of cellular senescence in epithelial cells by genetic complementation. A 226L 8/13 complementation assay was developed and used to determine the relative importance of the p53 and pRb pathways in the loss of growth potential upon inactivation of LT. It was shown that the 226L 8/13 conditional growth arrest could be complemented at the non-permissive temperature by inhibition of the p53 pathways by expressing GSE p53 or shRNAs against p53 and shRNAs targeting p21. Growth complementation by inhibition of the p53 pathway was highly efficient, even though slightly less efficient than reconstitution of LT activity, suggesting that the pRb pathway provides an additional proliferative barrier. Furthermore, it was shown that inhibition of the pRb pathway by expression of E1A and E2F-DB could overcome the conditional growth arrest of 226L 8/13 cells, but this was far less efficient than inhibition of the p53 pathway and only caused a proportion of cells to overcome the growth arrest. These results suggested that the p53 pathway is the main pathway for induction of senescence in 226L 8/13 cells, and that pRb provides an additional barrier to stop cells from proliferating.

Once it had been shown that 226L 8/13 cells could be used as a model to study cellular senescence, the murine ecotropic receptor was introduced into the cells and a single cell clone was selected for the screen, EcoR Clone 7. This clone was infectable with ecotropic viruses, highly conditional, i.e. it grew at the permissive temperature but arrested at the non-permissive temperature, and it readily overcame the conditional arrest by abrogation of the p53 pathway, and also by expression of E1A and E2F-DB. It

was then shown that EcoR Clone 7 reflected characteristics of the mixed population 226L 8/13 cells in respect to expression of epithelial markers and luminal and basal markers. Furthermore, it was shown that it reflected the growth characteristics of the mixed population 226L 8/13. Therefore, EcoR Clone 7 was a good model system to perform a loss of function shRNA screen.

Two loss of function shRNAmir screens were carried out in the conditional EcoR Clone 7 cells to identify mediators of cellular senescence in human breast epithelial cells, using the Open Biosystems pSM2c and the pGIPZ shRNAmir libraries.

From the pSM2c library screen, a total of 110 clones were ring-cloned from the 100 pools, and from these 110 clones 84 hairpins were identified. These led to the identification of 63 individual shRNA constructs. Out of these, 5 led to EcoR Clone 7 cells overcoming the conditional arrest in the secondary screen, the LOC219321 shRNA 53095, the Rab23 shRNA 115231, the LOC349975 shRNA 109096, the IRF-1 shRNA 133394, and the LOC346321 shRNA 145373.

From the 7 pGIPZ pools, a total of 22 candidate hairpins were identified, and in the secondary screen, 7 shRNAs led to EcoR Clone 7 cells overcoming the conditional arrest. These were the LOC343425 shRNA 25465, the FLJ31301 shRNA 68969, the LOC351347 shRNA 127153, the CCNL1 shRNA 40050, the BRCA1 shRNA 254609, the FOXA1 shRNA 16814, and the SPOPL shRNA 14455.

It was demonstrated that multiple hairpins targeting CCNL1, FOXA1, Rab23, SPOPL and IRF-1 were sufficient to bypass the conditional arrest of EcoR Clone 7 cells. Furthermore, knock-down of four of the target genes by the hairpins tested in the complementation assay was demonstrated. This was done by western blotting and quantitative RT-PCR for CCNL1, FOXA1, and Rab23 and by quantitative RT-PCR for SPOPL. Knock-down of IRF-1 was not demonstrated, but the fact that 5 individual IRF-1 shRNAs overcame the conditional arrest demonstrates that this was due to IRF-1 knock-down, not off-target effects. It was suggested that LOX might act downstream of IRF-1 to induce the growth arrest, and preliminary results support this hypothesis. Roles for IRF-1, Rab23, FOXA1 and CCNL1 in cell proliferation, senescence and



tumourigenesis have previously been suggested, supporting the results presented in this thesis.

In conclusion, the 226L 8/13 and EcoR Clone 7 cells were demonstrated to be good models to study senescence and to identify genes involved in this process in human breast epithelial cells. Several genes which mediate growth arrest were identified. These genes may represent mediators of cellular senescence in various cell types and might therefore play a role in the prevention of tumourigenesis. There is already significant evidence suggesting that FOXA1, Rab23, IRF-1 and CCNL1 play a role in cell proliferation, senescence and tumourigenesis, supporting the results presented in this thesis:

FOXA1 and Rab23 have been shown to inhibit growth of breast cancer cell lines, including MCF-7, MDA-MB-231, Bcap-37 and SKBR3 (Chao et al., 2009; Wolf et al., 2007). FOXA1 can activate the p27 promoter in conjunction with BRCA1 or alone (Williamson et al., 2006), and FOXA1 expression correlates with a good outcome in breast cancer, and was inversely related with tumour size (Albergaria et al., 2009; Habashy et al., 2008).

IRF-1<sup>-/-</sup> EFs can be transformed by expression of an activated Ras (Tanaka et al., 1994). IRF-1 has been shown to be required for Ras-induced growth suppression of myeloid cells (Passioura et al., 2005), and it has been shown to be inactivated or lost in several tumours; for example IRF-1 expression was shown to be lost in human breast-carcinoma (Doherty et al., 2001). IRF-1 has also been shown to regulate DNA damage-induced cell cycle arrest in cooperation with the tumour suppressor p53 through transcriptional activation of p21 (Tanaka et al., 1996).

CCNL1 has been shown to be down-regulated in certain types of breast cancer, for example DCIS (Abba et al., 2004) and ER positive breast cancers (Tripathi et al., 2008). Both these findings indicate that loss of CCNL1 might be involved in development of breast cancer. There is also evidence supporting a role for CCNL1 as a negative regulator of cell proliferation, as it has been shown to be down-regulated in dividing primary follicles compared to non-dividing primordial follicles, together with other genes involved in cell cycle control such as Wee1 and Lats2.

## 8.2 Future directions

### 8.2.1 Do DNA-SCARS cause 226L 8/13 cells to arrest at 38°C

Because the 226L 8/13 cells express hTERT, the senescence arrest at the non-permissive temperature cannot be caused by telomere shortening. It is unclear what causes the cells to arrest when the ts LT becomes inactive at the non-permissive temperature.

Rodier (Rodier et al., 2011) and colleagues showed the presence of DNA-SCARS in senescent cells, which are relatively stable DNA-damage foci that are distinct from transient damage foci (for example they lack the DNA repair proteins RPA and RAD51, single-stranded DNA and DNA synthesis, and they accumulate activated forms of the DDR mediators CHK2 and p53), and they demonstrated that SCARS are important for the senescence arrest. Rodier and colleagues showed that cells expressing SV40LT harboured senescence levels of DNA-SCARS but continued to proliferate, indicating that defective cell cycle checkpoints can uncouple DNA-SCARS from the senescence growth arrest. SCARS initiate senescence via DNA-damage response (DDR) signalling. 226L 8/13 cells express SV40LT, and it is therefore possible that the cells harbour SCARS when grown at the permissive temperature, and that when SV40 LT is inactivated at the non-permissive temperature, p53 and pRb become active and induce senescence. Therefore, 226L 8/13 cells grown at both 34°C and 38°C should be tested for the presence of DNA-SCARS, and the importance of DDR in 226L 8/13 cells should be analysed. The fact that the p53 pathway plays an important role in the induction of senescence in 226L 8/13 cells suggests that the arrest might be induced by DDR. The p53 pathway has been shown to play an important role in DNA-damage induced senescence (d'Adda di Fagagna et al., 2003; DiLeonardo et al., 1994; Herbig et al., 2004).

This leads to the question of why both hTERT and SV40LT were required to immortalize human breast luminal epithelial cells, whereas other groups have shown that introduction of hTERT alone is sufficient to immortalize several somatic cell types (Bodnar et al., 1998; Ouellette et al., 2000; Vaziri and Benchimol, 1998; Yang et al., 1999). Possibly, forcing these cells to divide *in vitro* could lead to the development of

DNA-SCARS, leading to cells senescing in the presence of a functional p53 and pRb pathway; this might explain the requirement of inhibition of the pRb and p53 pathway by SV40 LT in addition to introduction of hTERT. It has been suggested that when cells are cultured under adequate conditions hTERT is sufficient to immortalize; Herbert and colleagues found that primary HMECs can be immortalized by introduction of hTERT without abrogating the pRb/p16<sup>INK4</sup> pathway when cells were grown on feeder layers (Herbert et al., 2002). It would be interesting to test primary luminal epithelial cells whose life-span has been extended by the introduction of telomerase for the presence of DNA-SCARS, and if DDR is involved in the induction of senescence in these cells.

### 8.2.2 Saturation screens

As discussed in chapters 5.3 and 6.5, neither the pSM2c screen nor the pGIPZ screen were fully saturated. The equation  $\{\ln(1-0.99)/\ln(1-1/(\text{Library Size}))\}$  (<http://www.stanford.edu/group/nolan/screens/screens.html>) which was used to determine the number of infectious events required per pool only achieves 99% confidence that the entire library is screened, but it does not take into consideration that not every time that an shRNA which is able to abrogate the growth arrest is assayed leads to abrogation of the growth arrest. In regards to the pGIPZ p21 and p53 constructs which had been shown to achieve abrogation of the conditional arrest, it was observed that at an MOI of 0.1 – 0.3 only a proportion of cells overcame the conditional arrest. Therefore, to ensure that the screen is truly saturated, this percentage needs to be determined and considered when calculating the amount of infectious events required to achieve a saturated screen. Positive controls for the pSM2c library should be obtained to test what percentage of p21 and p53 shRNA infected cells overcome the conditional arrest, and this percentage should then be used to calculate the number of infectious events required to achieve a saturated screen. The screens could then be carried out with more infectious events to achieve saturation. But performing the screen with a very high amount of infectious events would also lead to a lot of false positives which would need to be eliminated.

### **8.2.3 Screening in a p16<sup>INK4a</sup> knock-down background**

Berns and colleagues (Berns et al., 2004) conditionally immortalised fibroblasts analogous to the 226L 8/13 cells to screen for mediators of cellular senescence. They found that knock-down of p16<sup>INK4a</sup> was insufficient to overcome the conditional growth arrest in their cells, but that knock down of p16<sup>INK4a</sup> in addition to knock-down of p53 gave stronger abrogation of the growth arrest compared to p53 knock-down alone. Therefore, they decided to screen for shRNAs that could abrogate the growth arrest in cells in which p16<sup>INK4a</sup> had been knocked down to facilitate the detection of mediators of senescence in their cell system. It should be established if knock-down of p16<sup>INK4a</sup> is insufficient to abrogate the growth arrest and if it improves abrogation of the growth arrest achieved by inhibition of the p53 pathway in EcoR Clone 7 cells. If so, a similar approach as that of Berns and colleagues could be used to facilitate the detection of mediators of senescence in EcoR Clone 7 cells.

### **8.2.4 Test target shRNAs in a Ras-induced senescence assay in HMECs**

Preliminary results (data not presented) suggest that some of the genes identified as mediators of senescence in EcoR Clone 7 cells, namely IRF-1, FOXA1 and Rab23, also play a role in inducing the OIS of VB3 cells, an assay developed by Borgdorff and colleagues (Borgdorff et al., 2010). VB3 cells are a cell line derived from a single-cell cloned HMEC, which harbour 4-hydroxy-tamoxifen (OHT)-inducible RasG12V and express hTERT and display a uniform senescence phenotype in response to an optimized dose of OHT. To prove statistical significance, an N of 3 is required, but due to a lack of time the experiment could not be repeated. Furthermore, background levels were very high in the experiment. This might have been due to residual GFP staining (from the pGIPZ constructs, most GFP is degraded by HCl treatment during BrdU staining) interfering with the BrdU signal (antibody Clone MoBU-1 conjugated with green fluorescent Alexa Fluor ® 488), and to overcome this problem, another fluorochrome which is not excited at the green spectrum should be used. The background levels could also have been due to levels of OHT being too low, therefore higher OHT levels should also be tested in the assay.

It might be more feasible to follow the approach used by Borgdorff and colleagues (Borgdorff et al., 2010), who optimised the OIS assay in VB3 cells using the Silencer whole genome synthetic siRNA library from Ambion (Austin, TX, USA). Constructs targeting the genes identified as mediators of senescence in EcoR Clone 7 cells could be obtained from this library and tested in the VB3 cell assay following the protocol optimised by Borgdorff and colleagues.

### **8.2.5 Test if silencing of target genes in conjunction with hTERT immortalises primary luminal epithelial cells**

Mike O'Hare and colleagues found that expression of hTERT was insufficient to immortalise human breast luminal epithelial cells, and that they underwent very few population doublings even upon introduction of hTERT. It could be tested if silencing of the target genes identified in the screen could immortalise or extend the proliferative potential of primary luminal epithelial cells in conjunction with hTERT. Particularly IRF-1, as it has been shown that IRF-1<sup>-/-</sup> EFs can be transformed by expression of an activated Ras (Tanaka et al., 1994) and IRF-1 has been shown to be required for Ras-induced growth suppression of myeloid cells (Passioura et al., 2005).

### **8.2.6 Expression of target genes in breast cancer cell lines**

The expression of genes which have been shown to mediate the EcoR Clone 7 growth arrest could be determined in breast cancer cell lines. Protein has already been harvested from several human breast cancer cells lines, including MCF-7, PMC42, MDA-MB-231, MDA-MB-468, ZR75, and T47D. If these genes are not expressed or only expressed at low levels in these cell lines, then expression constructs could be made and introduced into such cell lines to analyse if forced expression of these genes could induce growth arrest.

Exogenous expression of FOXA1 has been shown to inhibit growth of the breast cancer cell lines MCF-7, MDA-MB-231, and SKBR3; furthermore, increased FOXA1 levels lead to a reduction in growth stimuli (Wolf et al., 2007).

Zeng and colleagues found that Rab23 overexpression inhibited the proliferation of some breast cancer cell lines, namely MDA-MB-231, Bcap-37 and MCF-7 when measured by colony formation assay, BrdU labelling and MTT assay; Rab23 silencing lead to increased proliferation (Chao et al., 2009).

### **8.2.7 Importance of the pRb pathway**

Inhibition of the p53 pathway by p53 GSE, p53 shRNA or p21 shRNA was very efficient in abrogating the growth arrest of 226L 8/13 cells, whereas inhibition of the pRb pathway by E1A and E2F-DB only bypassed the conditional arrest in a small proportion of the cells. Nevertheless, inhibition of p53 was not as efficient in abrogating the growth arrest as introduction of wt LT antigen. As LT antigen inactivates both the p53 and the pRb pathway, this finding suggests that pRb provides an additional barrier to stop cells from proliferating. It should be tested if inhibition of the pRb pathway in addition to the p53 pathway would increase abrogation of the growth arrest of 226L 8/13 cells.

As mentioned above, only a subpopulation of 226L 8/13 cells overcame the conditional growth arrest upon infection with E1A and E2F-DB. It would be interesting to analyse this subpopulation by expression profiling or deep sequencing and identify and if these cells carry epigenetic changes or mutations which enable abrogation of the arrest by inhibition of the pRb pathway. It has been shown that a lot of tumour suppressors or activated oncogenes need additional mutations for cells to become immortal.

Knock-down of the targets identified in the screen also only abrogated the growth arrest in a subpopulation of EcoR Clone 7 cells. Therefore, such cells which can overcome the growth arrest due to knock-down of these targets might also harbour epigenetic changes or mutations which enable them to do so. If the EcoR Clone 7 growth arrest is caused by DNA-SCARS, such changes might be in the DDR pathway.

### 8.2.8 IRF-1 and its role in epithelial senescence

IRF-1 knock-down appeared to achieve the strongest abrogation of the EcoR Clone 7 growth arrest out of all the targets identified in the screen. As discussed in chapter 7.3.5.1 IRF-1 has been shown to be involved in the cell cycle and induction of senescence. It has been demonstrated that there is an IRF response element in the LOX promoter, and that expression of LOX cDNA can suppress the transformed phenotype of Ras-transformed IRF-1 null embryonic fibroblasts, inhibiting colony formation (Tan et al., 1996). Therefore, the effect of knock-down of LOX in the EcoR Clone 7 growth arrest was tested and it was shown that two LOX hairpins, namely 134020 and 348880, gave rise to several healthy looking colonies of growing cells, suggesting that LOX knock-down might be sufficient to overcome the EcoR Clone 7 growth arrest. Knock-down of LOX by the hairpins should be determined. Furthermore, if LOX shRNAs abrogate the EcoR Clone 7 growth arrest, this suggests that LOX might act downstream of IRF-1 in senescence in EcoR Clone 7 cells, but this would need to be investigated further. It could be analysed if IRF-1 binds to and activates the LOX promoter in EcoR Clone 7 cells when shifted to the non-permissive temperature. Furthermore, a constitutively active LOX could be expressed in EcoR Clone 7 cells and the ability of IRF-1 shRNAs to abrogate the growth arrest could be tested. If IRF-1 knock-down cannot abrogate the growth arrest in the presence of a constitutively active LOX, that would suggest that it acts via LOX to mediate growth arrest in EcoR Clone 7 cells.

### 8.3 Final remarks

Cellular senescence is a potent anti-tumour mechanism. Evidence for this is the accumulation of cells expressing senescent markers *in vivo* in benign tumours, preventing the emergence of carcinoma (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Gray-Schopfer et al., 2006; Michaloglou et al., 2005). In contrast to normal somatic cells, cancer cells have the potential to proliferate indefinitely and this acquisition of an infinite proliferative potential has been proposed to be one of the six key events required for malignant transformation (Hanahan and Weinberg, 2011).

On the other hand, senescent cells accumulate in multiple tissues and they are often associated with pathologies, such as atherosclerosis. The accumulation of senescent cells may impair tissue repair and renewal due to a depletion of stem and progenitor cells. The secretory phenotype of senescent cells, SASP, includes proteins that are involved in tissue environment (Acosta et al., 2008; Cristofalo and Pignolo, 1996; Kuilman et al., 2008), and can therefore alter functionality and structure of tissue which could contribute to the ageing phenotype. Furthermore, senescent cells, in contrast to their tumour-protective function, can promote carcinogenesis in vivo due to SASP and secretion of matrix degrading enzymes (Coppe et al., 2008; Krtolica et al., 2001; Kuilman and Peeper, 2009).

Genes identified in this study may have prognostic and/or diagnostic value in the context of cancer biology. Furthermore, there is evidence that the induction of senescence is critical for the efficacy of chemotherapeutic agents (Berns, 2002; Chang et al., 2002; Gewirtz et al., 2008; Zheng et al., 2004). Therefore, elucidating pathways underlying senescence will be important for the development of novel chemotherapeutic agents.



## 9 References

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